TRANSMITTAL OF APPEAL BRIEF

Docket No. GNN-018CP

In re Application of: Joseph SYPEK *et al.*

Application No.	Filing Date	Examiner	Group Art Unit
09/805800-Conf. #2859	March 13, 2001	P. Gambel	1644

Invention: USE OF RAPAMYCIN AND AGENTS THAT INHIBIT B7 ACTIVITY IN IMMUNOMODULATION

TO THE COMMISSIONER OF PATENTS:

Transmitted herewith is the Appeal Brief in this application, with respect to the Notice of Appeal filed: June 4, 2004 .

The fee for filing this Appeal Brief is $ 500.00 .

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Dated: January 3, 2005

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Docket No.: GNN-018CP
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Joseph Sypek, *et al.*	Group Art Unit: 1644
Serial No.: 09/805,800	Examiner: P. Gambel
Filed: March 13, 2001	
For: *Use of Rapamycin and Agents That Inhibit B7 Activity in Immunomodulation*	
Attorney Docket No.: GNN-018CP	

MS Appeal Brief - Patents
Commissioner for Patents
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APPEAL BRIEF

As indicated in the Notice of Appeal filed on June 4, 2004, Appellants hereby appeal the final decision of the Examiner in the above-identified application rejecting the subject matter of the pending claims. For the reasons set forth in this brief, Appellants respectfully request the Board of Patent Appeals and Interferences to reverse the Examiner's final rejection of the claimed subject matter.

I. REAL PARTY IN INTEREST

The real party in interest in the above-identified application is Genetics Institute, Inc.

II. RELATED APPEALS AND INTERFERENCES

No other appeals or interferences are known to Appellants, Appellants' legal representative, or the assignees which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1, 4, 5, and 7-13 are pending in this application. Claims 2, 3, and 6 have been previously canceled.

Claims 1, 4, 5, and 7-13 are on appeal and are set forth in Appendix A.

IV. STATUS OF THE AMENDMENTS

A Notice of Appeal was filed on June 4, 2004. All prior amendments have been entered. A Request for an appropriate Extension of Time is being filed with this Appeal Brief.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The instant invention is based, at least in part, on the finding that agents that decrease co-stimulatory signals to T cells are more efficient in reducing symptoms of autoimmune disease when used in combination with Rapamycin or Rapamycin-like compounds. Accordingly, the invention provides improved methods of downmodulating immune responses by a cell expressing a B7 molecule with a combination of at least one antibody which binds to at least one B7 molecule and a Rapamycin compound (see, *e.g.,* page 5, lines 3-6).

One aspect of the invention provides a method for downmodulating an immune response comprising contacting immune cells from a subject with a Rapamycin compound in combination with at least two antibodies, wherein each of said antibodies binds to a different B7 molecule selected from the group consisting of B7-1, B7-2, B7-H1, or B7RP-1 (see, *e.g.,* page 5, lines 6-8). In one embodiment, the step of contacting is performed *in vivo* (see, *e.g.,* page 5, line 10).

In another aspect of the invention, a method for downmodulating an immune response in a subject having an autoimmune disorder is provided, comprising contacting immune cells from the subject with a Rapamycin compound in combination with at least two antibodies, wherein each of said antibodies binds to a different B7

molecule selected from the group consisting of B7-1, B7-2, B7-H1, or B7RP-1(see, *e.g.,* page 6, lines 8-10). In one embodiment, the autoimmune disorder is systemic lupus erythematosis (see, *e.g.,* page 5, lines 10-11).

In one aspect of the invention, a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis is provided, comprising administering to the subject an antibody that binds to B7-1, an antibody that binds to B7-2, and a Rapamycin compound, wherein the antibody that binds to B7-1 and the antibody that binds to B7-2 are administered over at least one short course of therapy (see, *e.g.,* page 10, lines 26-28).

In another aspect of the invention, a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis is provided, comprising administering to the subject an antibody that binds to B7-1, an antibody that binds to B7-2, and a Rapamycin compound, wherein the Rapamycin compound is administered over at least one intermediate course of therapy (see, *e.g.,* page 10, lines 28-32).

In yet another aspect of the invention, a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis is provided, comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered over at least one extended course of therapy (see, *e.g.,* page 10, line 32, through page 11, lines 1-4).

In one aspect of the invention, a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis is provided, comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered according to an early dosing regimen (see, *e.g.,* page 11, lines 5-8).

In another aspect of the invention, a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis is provided, comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered according to a late dosing regimen (see, *e.g.,* page 11, lines 8-10).

In one aspect, the invention provides a method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising

administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, a Rapamycin compound, and an immunosuppressing agent, wherein said immunosuppressing agent is selected from the group consisting of FK506, Cyclosporine A and cyclophosphamide (see, *e.g.*, page 41, lines 15-18).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Appellants present the following issues for review:

1. Whether claims, 1, 4, 5, and 7-13 are properly rejected under 35 U.S.C. §103(a) as obvious over Co, *et al.* (US 2002/0176855 A1) in view of deBoer, *et al.* (US Patent No. 5,747,034), Cottens, *et al.* (WO 95/16691), and Strom, *et al.* (Therapeutic Immunology, Austen *et al.*, (Ed.) Blackwell Science, Cambridge, MA 1996).

VII. ARGUMENTS

1. Rejection of Claims , 2, 4, 5, and 7-12 Under 35 U.S.C. §103(a)

The Examiner rejects claims 1, 4, 5, and 7-13 under 35 U.S.C. §103(a) as obvious over Co, *et al.* (US 2002/0176855 A1) (Appendix B) in view of deBoer, *et al.* (US Patent No. 5,747,034) (Appendix C), Cottens, *et al.* (WO 95/16691) (Appendix D), and Strom, *et al.* (Therapeutic Immunology, Austen *et al.*, (Ed.) Blackwell Science, Cambridge, MA 1996). (Appendix E).

It is the Examiner's position that:

> [t]he primary and secondary references provide clear teachings of combining immunosuppressives in therapeutic regimens to inhibit the immune response including antibodies and [R]apamycin . . . . . Therefore, the prior art provides motivation and expectation of success in combining immunosuppressives in therapeutic regimens including the expected advantage of additive-synergistic effects and reducing toxicity of certain immunosuppressives.

The Examiner also states that:

> Given the teachings of the prior art to combine anti-B7-1 and anti-B7 antibodies alone or in combination with other immunosuppressive therapy to inhibit immune responses, including therapeutic regimens of treating SLE alone in conjunction with the know[n] use of [R]apamycin to treat SLE alone or in combination with other immunosuppressive antibodies, including anti-B7 antibodies; one of ordinary skill in the art at the time the invention was made would have been motivated to combine anti-B7-1 and anti-B7-2 antibodies with [R]apamycin to inhibit immune responses in various therapeutic regiments including the

> treatment of SLE at the time the invention was made. The various dosing regiments encompasse[d] by the instant claims were obvious at the time the invention was made, given that it was well known and practice[d] at the time the invention was made to provide immunosuppressive therapy based upon the condition and needs of the patient, as evidenced by the teachings of the prior art.

For the reasons set forth below, it is Appellants' position that in view of the teachings in the cited art, and the general knowledge of the art at the time of filing, one of ordinary skill in the art would not have been motivated to combine the teachings of the cited art to produce the claimed invention. A case of *prima facie* obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. Appellants contend that the Examiner has failed to present a *prima facie* case of obviousness as there is nothing in any of the cited prior art references, or in the knowledge of the art generally, that would suggest to one of skill in the art to combine the references to arrive at the claimed invention. Moreover, even if the references are combined, there is no teaching or suggestion in the references to downmodulate an immune response, *e.g.,* an autoimmune response, by contacting immune cells from a subject with a Rapamycin compound ***and*** at least two antibodies that bind to different B7 molecules, and certainly no expectation of success in doing so.

The present invention teaches methods of downmodulating an immune response with a combination of an anti-B7-1 antibody, an anti-B7-2 antibody ***and*** Rapamycin. In contrast, Co, *et al.* (Appendix B) teach that immune-related or autoimmune diseases and disorders can be treated using an antibody specific to B7-2, and that treatment of these diseases may be facilitated by co-administration of an anti-B7-2 antibody with an anti-B7-1 antibody, or antibodies to the corresponding receptors, CD28 and CTLA-4. Co, *et al.* further teach that methods of treatment also involve co-administration of a humanized anti-B7-2 antibody ***or*** a humanized anti-B7-1 antibody with other standard therapy drugs (see page 10, column 2).

> Treatment of these diseases may be facilitated with coadministration of an anti-B7-2 antibody, including chimeric and humanized versions thereof, with an anti-B7-1 antibody or antibodies to the corresponding receptors, CD28 and CTLA-4.

Therefore, Co, *et al.* do not teach or suggest methods of treating any immune disease or disorder with a combination of an anti-B7-1 antibody, an anti-B7-2 antibody ***and*** Rapamycin as presently claimed. In fact, nothing in the teachings of Co, *et al.* suggests that co-administration of two antibodies that bind two different B7 molecules would be advantageous, let alone the combination of those two antibodies and Rapamycin, as claimed. Moreover, Co *et al.* do not teach, or even suggest, the administration of Rapamycin. Indeed, the very absence of Rapamycin in the list of "standard therapy drug" examples provided by Co, *et al.* clearly implies that, as experts in the field, they did not consider using Rapamycin in combination with one B7 antibody, let alone two B7 antibodies, as presently claimed.

deBoer, *et al.* (Appendix C) disclose that the combination of anti-B7-1 antibodies and cyclosporine results in tolerance, a surprising discovery since it was previously suggested that cyclosporine ***inhibits*** anergy induction (column 5, eighth paragraph). deBoer, *et al.* teach that co-administration of a B7-1 antibody with cyclosporine A "completely blocks" T cell activation (column 25, second paragraph) and successfully inhibits T cell proliferation ***in the absence of blocking agents for B7-2*** (*e.g.*, see column 28, lines 22-28). deBoer, *et al.* further state that

> [g]iven that both B7-1 and B7-2 may provide the co-stimulatory signal to T cells for the production of IL-2 (a molecule that inactivates anergy genes), ***it is surprising that blocking only B7-1 in combination with cyclosporine results in T cell tolerance***. This may be explained by the fact that signal transduction after cross-linking with CD28 results in two independent signaling pathways, one being Cyclosporine-sensitive and one being Cyclosporine-insensitive. It may be that ***signal transduction after interaction of CD28 with B7-2 is mediated by the Cyclosporine-sensitive pathway***. (column 6, lines 27-33). (emphasis added).

Thus, Applicants respectfully submit that one of ordinary skill in the art would conclude from the teachings of deBoer, *et al.* that the administration of anti-B7-2 antibodies is unnecessary when cyclosporine is administered in combination with anti-B7-1 antibodies, *i.e.*, the combination of anti-B7-1 antibodies and cyclosporine would be equivalent to the combination of anti-B7-1 and anti-B7-2 antibodies because cyclosporine acts as a ***substitute*** for anti-B7-2 antibodies. deBoer, *et al.* therefore actually teaches away from the claimed methods. In addition, while deBoer, *et al.* suggest that other immunosuppressive agents, including Rapamycin, might be used in combination with B7-1 antibodies, they fail to provide any teaching whatsoever that

any of these other immunosuppressive agents would be as effective as cyclosporine, let alone suggest the co-administration of Rapamycin with two anti-B7 antibodies, as presently claimed.

Accordingly, there is, therefore, no motivation to combine the teachings of Co, *et al.* and deBoer, *et al.* to arrive at the claimed methods. Moreover, even if the teachings of Co, *et al.* and deBoer, *et al.* are combined, they merely teach that immune disorders can be treated by inhibiting the B7 pathway using one of the following combinations (1) anti-B7-1 and anti-B7-2 antibodies (not Rapamycin); (2) anti-B7-2 antibodies and standard therapy drugs; or (3) anti-B7-1 and cyclosporine (as a substitute for anti-B7-2). Indeed, one of ordinary skill in the art, when presented with the combined teachings of deBoer, *et al.* and/or Co, *et al.* in their entirety would not have been motivated to use a combination of anti-B7-1 antibodies, anti-B7-2 antibodies and Rapamycin to treat an immune disorder, let alone be able to predict that this combination would result in improved results as demonstrated by Appellants (*e.g.*, an increased level of survival in a clinically relevant animal model when compared to the level of survival in animals treated with only B7-1 and B7-2 antibodies (see Example 2, page 49, lines 1-9, Example 3, page 49, lines 11-22, and Figure 4)).

The teachings of Cottens, *et al.* (Appendix D) and Strom, *et al.* (Appendix E), either alone or in combination, do not cure the deficiencies of Co, *et al.* and deBoer, *et al.* Cottons, *et al.* merely teach novel Rapamycin derivatives that have "an improved pharmacological profile over Rapamycin, exhibit greater stability and bioavailability, allow for greater ease in producing formulations, and are more potent immunosuppressants" (page 2, second paragraph). Cottens, *et al.* generally suggest that the novel compounds might be used in combination with other immunosuppressive drugs or immunosuppressive monoclonal antibodies, but provide no specific teaching of any combination that is effective in inducing anergy. Strom, *et al.* merely teach a multi-tiered approach to immunosuppressive therapy. Strom, *et al.* further disclose that the majority of basic protocols involve a combination of cyclosporine or FK506 plus corticosteroids with or without azathioprine, and suggest that anti-lymphocyte globulin or OKT3 might also added to reduce the dose of cyclosporine required (page 454). Thus, Strom, *et al.* merely include Rapamycin in their general list of immunosuppressants with nothing more.

In short, none of the cited references alone or in combination suggest the use of a combination of Rapamycin with at least two B7 antibodies as presently claimed. At best, the cited references might be viewed as generally providing the suggestion to try various combinations of immunosuppressive antibodies and immunosuppressive agents. Indeed, Appellants respectfully submit that the Examiner has failed to point to any teaching in the Co, *et al.*, deBoer, *et al.* and/or Cottens, *et al.* and Strom, *et al.* references that would compel one of ordinary skill in the art to make the claimed invention. As the Board is well aware, the prior art must suggest "to those of ordinary skill in the art that they ***should*** make the claimed composition or device, or carry out the claimed process" and [b]oth the suggestion and the reasonable expectation of success ***must be founded in the prior art, not in the applicant's disclosure*** (emphasis added)." *In re Dow Chemical Co.* 837 F.2d 469, 473, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988).

Appellants further rely on to *Arkie Lures v. Larew Tackle*, 119 F.3d 953, 43 U.S.P.Q. 2d 1294 (Fed. Cir. 1997) to support their position. In *Arkie Lures*, the Larew invention was directed to a "salt-impregnated fishing lure." In that case, the CAFC overturned the district court's finding of obviousness. The CAFC agreed that "[t]he use of salty bait to catch fish was known, [and] plastisol lures were known." *Id* at page 956. However, the CAFC found that although the literature on "fishing lures is apparently quite extensive, but despite the long use of salty lures and plastic lures, no reference was cited that showed or suggested this combination." *Id* at page 956. The CAFC continued and stated that "[t]he evidence showed the complexity of the plastic fishing lure art. Those in the field of the invention viewed Larew's invention not as a simple concept of adding salty taste to a known lure, but as a complex combination requiring experience of fishing and fishing lures and the technology of plastics." *Id* at page 957. The court further stated that:

> No prior art showed or suggested the combination of a plastisol lure with salt, although the prior art was extensive as to the separate elements, and suggested including organic attractants in plastic lures. . . . The question is not whether salt "could be used," as the district court concluded, but whether it was obvious to do so in light of all the relevant factors. . . ***It is insufficient to establish obviousness that the separate elements of***

> ***the invention existed in the prior art, absent some teaching or suggestion, in the prior art, to combine the elements.*** Indeed, the years of use of salty bait and of plastic lures, without combining their properties, weighs on the side of unobviousness of the combination (emphasis added).

Id at pages 957 and 958.

Similar to the situation in the *Arkie Lures* case, even if the prior art contained the separate elements of the presently claimed methods, these individual teachings are insufficient to establish the obviousness of the claimed invention absent some teaching or suggestion in the art to combine and modify the teachings of those references to arrive at the claimed invention.

In further support of their position, Appellants point to the CAFC decision in *In re Rouffet*, (149 F.3d 1350) (Fed. Cir. 1998)). Rouffet filed a patent application directed to technology to reduce signal transmission and receptor interruptions in the transmission signals from satellites. Rouffet taught changing the shape of the beam transmitted by the satellite's antenna to a fan-shaped beam. The Examiner rejected Rouffet's claims as unpatentable over U.S. Patent number 5,199,672 (King) in view of U.S. Patent number 4,872,015 (Rosen) and a report titled "A Novel Non-Geostationary Satellite Communications System" (Ruddy). The CAFC found that:

> [although] the board did not err in finding that the combination of King, Rosen, and Ruddy contains all of the elements claimed in Rouffet's application. . .the Board reversibly erred in determining that one of skill in the art would have been motivated to combine these references in a manner that rendered the claimed invention obvious. Indeed, ***the Board did not identify any motivation to choose these references for combination.*** (emphasis added).

Id at page 1357.

Similarly, it is Appellants' position that the Examiner has failed to point to ***any motivation*** to combine the cited prior art references, let alone combine them in performing the claimed method. In *Rouffet* the CAFC continued:

> [b]ecause the Board did not explain the specific understanding or principle within the knowledge of a skilled artisan that would motivate one with no knowledge of Rouffet's invention to make the

> combination, this court infers that the examiner selected these references with the assistance of hindsight. This court forbids the use of hindsight in the selection of references that comprise the case of obviousness. See In re Gorman, 933 F.2d 982, 986, 18 U.S.P.Q. 2D (BNA) 1885, 1888 (Fed Cir. 1991). ***Lacking a motivation to combine references, the Board did not show a proper prima facie case of obviousness.*** This court reverses the rejection over the combination of King, Rosen, and Ruddy.

In at page 1357.

Additional support for Appellants' position that the claimed invention is not obvious is found in *In re Vaeck* (*In re Vaeck* 947 F.2d 488. (Fed. Cir. 1991)). In *Vaeck* the invention was drawn to "a chimeric (*i.e.*, hybrid) gene comprising (1) a gene derived from a bacterium of the *Bacillus* genus whose product is an insecticidal protein, united with (2) a DNA promoter effective for expressing the Bacillus gene in a host cyanobacterium, so as to produce the desired insecticidal protein (footnote omitted)." *Id* at page 490. The prior art was applied in various combinations against the claims. The primary reference (Dzelzkalns) taught the expression of a chimeric gene comprising a chloroplast promoter sequence fused to a gene encoding the enzyme chloramphenicol acetyl transferase (CAT) in cyanobacteria. The secondary references taught, *inter alia,* "expression of genes encoding certain *Bacillus* insecticidal proteins" in other host cells; "the initiation specificities *in vitro* of DNA-dependent RNA polymerases purified from two different species of cyanobacteria (footnote omitted);" and "host-vector systems for gene cloning in the cyanobacterium." *Id* at page 491. The Examiner's position was that:

> it would have been obvious to one of ordinary skill in the art to substitute the *Bacillus* genes [which had been expressed in heterologous hosts in the teachings of the prior art] for the CAT gene in the vectors of Dzelzkalns in order to obtain high level expression of the *Bacillus* genes in the transformed cyanobacteria. The Examiner further contended that it would have been obvious to use cyanobacteria as heterologous hosts for expression of the claimed genes due to the ability of cyanobacteria to serve as transformed hosts for the expression of heterologous genes.

Id at page 492. The CAFC disagreed with the PTO's position and found that the teachings of the prior art cited were not sufficient to support the interchangeability of bacteria and cyanobacteria as host organisms for the expression of heterologous insecticidal proteins. The CAFC stated that "there is no suggestion in Dzelzkalns, the primary reference cited against all claims, of substituting in the disclosed plasmid a structural gene encoding *Bacillus* insecticidal proteins for the CAT gene utilized for selection purposes. The expression of antibiotic resistance-conferring genes in cyanobacteria, without more, does not render obvious the expression of unrelated genes in cyanobacteria." *Id* at page 493. The court further stated that while the prior art disclosed "expression of *Bacillus* genes encoding insecticidal proteins in certain transformed bacterial hosts, nowhere do these references disclose or suggest expression of such genes in transformed cyanobacterial hosts. . . . [w]hile it is true that bacteria and cyanobacteria are now both classified as prokaryotes, that fact alone is not sufficient to motivate the art worker as the PTO contends." *Id* at pages 493 and 494.

The CAFC contrasted its findings in *In re Vaeck* with those in *In re O'Farrell* stating "[i]n contrast with the situation in *O'Farrell*, the prior art in this case offers no suggestion, explicit or implicit, of the substitution that is the difference between the claimed invention and the prior art." *Id* at page 495. In *O'Farrell* the invention was directed to a "method for producing a predetermined protein in a stable form in a transformed host species of bacteria." *In re O'Farrell* 853 F.2d 894. 1988. 7 U.S.P.Q. 2d (BNA) 1673. The prior art (Polisky) taught a previous attempt to "control the expression of cloned heterologous genes inserted into bacteria." *Id* at page 899. The prior art differed from the claim at issue, however, because it taught a method of expressing "a segment of DNA from a frog that coded for ribosomal RNA," which is normally not translated into protein. Although ribosomal RNA is not normally translated into protein, the court found that in the prior art publication by Polisky the authors were "obviously interested in using their approach to make heterologous proteins in bacteria." *Id* at page 900. The CAFC referred to the Polisky paper which stated:

> In fact, we have recently observed that induced cultures of pBGP123 contain elevated levels of [beta]-galactosidase of higher subunit molecular weight than wild-type enzyme (P. O'Farrell, unpublished experiments). We believe this increase results from

> translation of Xenopus [frog] RNA sequences covalently linked to [messenger] RNA for [beta]-galactosidase, resulting in a fused polypeptide.

Id at page 900 (quoting from Polisky *et al.* at page 4904). The court stated that "[t]he authors of the Polisky paper ***explicitly pointed out*** that if one were to insert a heterologous gene coding for a protein into their plasmid, it should produce a 'fused protein' consisting of a polypeptide made of beta-galactosidase plus the protein coded for by the inserted gene, joined by a peptide bond into a single continuous polypeptide chain." (emphasis added) *Id* at page 901. The court also referred to a passage in the Polisky reference, wherein the authors stated that "[i]f an inserted sequence contains a ribosome binding site that can be utilized in bacteria, production of high levels of a read-through transcript might allow for extensive translation of a functional eukaryotic polypeptide." *Id* at page 901 (quoting from Polisky *et al.*). The CAFC upheld the PTO decision that the claims in *O'Farrell* were obvious over Polisky because:

> virtually everything in the claims was present in the prior art. . . . The main difference is that in Polisky the heterologous gene was a gene for ribosomal RNA while the claimed invention substitutes a gene coding for a predetermined protein. . . .Nevertheless, Polisky mentioned preliminary evidence that the transcript of the ribosomal RNA gene was translated into protein. Polisky further predicted that if a gene that codes for a protein were to be substituted for the ribosomal RNA gene, 'a read-through transcript might allow for extensive translation of a functional eukaryotic polypeptide.' ***Thus, the prior art explicitly suggested the substitution that is the difference between the claimed invention and the prior art, and presented preliminary evidence suggesting that the method could be used to make proteins***. (emphasis added)

Id at 901.

It is Appellants' position that, as in *In re Vaeck*, there is no teaching, either explicit or implicit, in any of the references cited by the Examiner, which would have impelled one of ordinary skill in the art to make the instantly claimed invention and at most, the teachings of the cited references merely provide the motivation to test any and all combinations of immunosuppressive antibodies and immunosuppressive agents.

Appellants submit that the Examiner has used Appellants' invention as a blueprint to combine the aforementioned references. As the Board is well aware, the

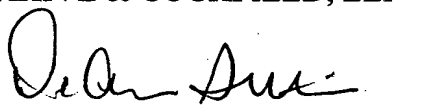
use of hindsight in the selection of references is forbidden. (See *In re Gorman*, 933 F.2d 982, 986, 18 U.S.P.Q. 2D (BNA) 1885, 1888 (Fed. Cir. 1991)). Appellants respectfully submit that the art cited by the Examiner is directed to individual elements of Appellants' invention, and there is no teaching in the references or the known art at the time of filing that would motivate one skilled in the art to combine the references. The Examiner has, therefore, improperly relied on hindsight obtained from Appellants' own invention to combine the cited references.

Moreover, even if the Board finds that the motivation existed to combine the teachings of the cited references, Appellants submit that the combined teachings of the references do not teach each and every element of the claimed methods. In particular, none of the references, either alone or in combination, teach methods of downmodulating an immune response utilizing a ***Rapamycin compound*** in combination with ***at least two antibodies*** that bind to ***different B7 molecules***.

In summary, Appellants contend that the Examiner has improperly relied on hindsight reconstruction obtained from Appellants' invention in combining the cited references, and as such, has failed to provide a *prima facie* case of obviousness. Moreover, Appellants submit that even if combined, the cited references do not teach, suggest or enable the claimed methods presently on appeal. Appellants therefore request that the Board withdraw the Examiner's rejection of the claims under 35 U.S.C. §103(a).

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



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Dated: January 3, 2005

APPENDIX A

1. (Previously Presented) A method for downmodulating an immune response comprising contacting immune cells from a subject with a Rapamycin compound in combination with at least two antibodies, wherein each of said antibodies binds to a different B7 molecule selected from the group consisting of B7-1, B7-2, B7-H1, or B7RP-1.

2. (Canceled)

3. (Canceled)

4. (Previously Presented) The method of claim 1, wherein the step of contacting is performed *in vivo*.

5. (Previously Presented) A method for downmodulating an immune response in a subject having an autoimmune disorder comprising contacting immune cells from the subject with a Rapamycin compound in combination with at least two antibodies, wherein each of said antibodies binds to a different B7 molecule selected from the group consisting of B7-1, B7-2, B7-H1, or B7RP-1.

6. (Canceled)

7. (Previously Presented) The method of claim 5, wherein the autoimmune disorder is systemic lupus erythematosis.

8. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising administering to the subject an antibody that binds to B7-1, an antibody that binds to B7-2, and a Rapamycin compound, wherein the antibody that binds to B7-1 and the antibody that binds to B7-2 are administered over at least one short course of therapy.

9. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising

administering to the subject an antibody that binds to B7-1, an antibody that binds to B7-2, and a Rapamycin compound, wherein the Rapamycin compound is administered over at least one intermediate course of therapy.

10. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered over at least one extended course of therapy.

11. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered according to an early dosing regimen.

12. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, and a Rapamycin compound, wherein said Rapamycin compound is administered according to a late dosing regimen.

13. (Previously Presented) A method for downmodulating an immune response in a subject suffering from systemic lupus erythematosis comprising administering to said subject an antibody that binds B7-1, an antibody that binds B7-2, a Rapamycin compound, and an immunosuppressing agent, wherein said immunosuppressing agent is selected from the group consisting of FK506, Cyclosporine A and cyclophosphamide.

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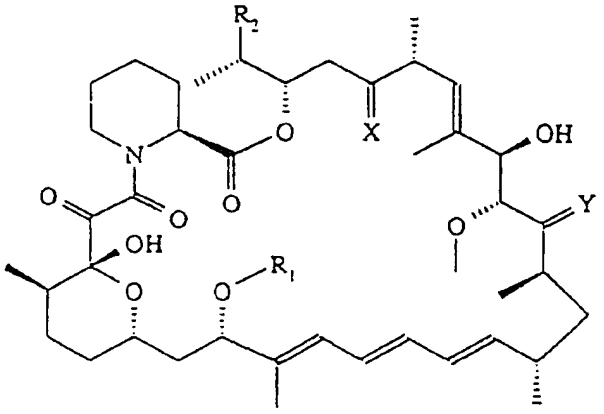
(57) Abstract

Novel demethoxy derivatives of rapamycin of formula (I) are found to have pharmaceutical utility, particularly as an immunosuppressants. In formula (I) R_2 = formula (II) or formula (III), X, Y, R_1, R_3, R_3, R_4, R_5 are as defined in the application.



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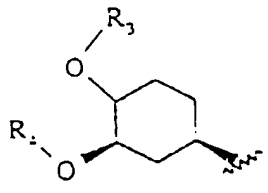

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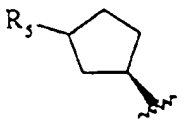

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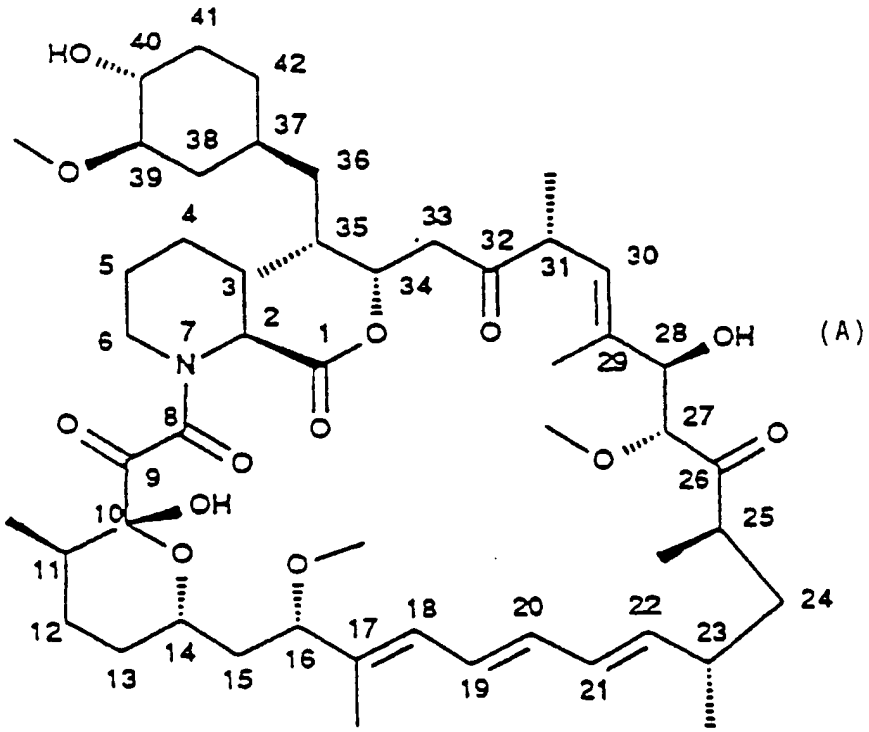
RAPAMYCIN DERIVATIVES USEFUL AS IMMUNOSUPPRESSANTS.

This invention comprises novel demethoxy derivatives of rapamycin, such derivatives having pharmaceutical utility, especially as immunosuppressants.

Rapamycin is a known macrolide antibiotic produced by Streptomyces hygroscopicus, having the structure depicted in Formula A:



See, e.g., McAlpine, J.B., et al., J. Antibiotics (1991) 44: 688; Schreiber, S.L., et al., J. Am. Chem. Soc. (1991) 113: 7433; US Patent No. 3 929 992. (There have been various numbering schemes proposed for rapamycin. To avoid confusion, when specific

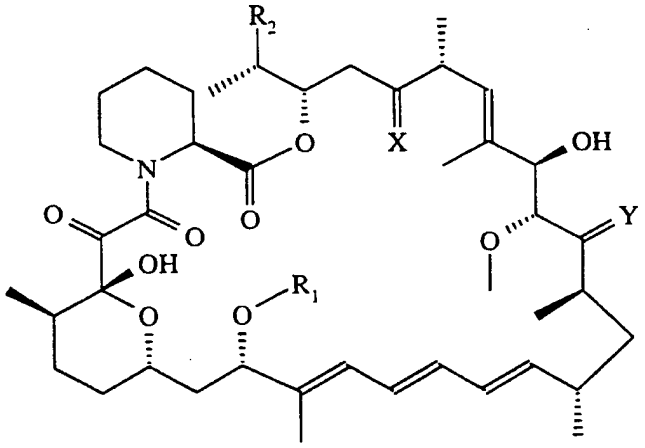
rapamycin derivatives are named herein, the names are given with reference to rapamycin using the numbering scheme of formula A.) Rapamycin is an extremely potent immunosuppressant and has also been shown to have antitumor and antifungal activity. Its utility as a pharmaceutical, however, is restricted by its very low and variable bioavailability as well as its high toxicity. Moreover, rapamycin is highly insoluble, making it difficult to formulate stable galenic compositions. Numerous derivatives of rapamycin are known. Certain 16-O-substituted rapamycins are disclosed in WO 94/02136, the contents of which are incorporated herein by reference. 40-O-substituted rapamycins are described in, e.g., in US 5 258 389 and PCT/EP 93/02604 (O-aryl and O-alkyl rapamycins); WO 92/05179 (carboxylic acid esters), US 5 118 677 (amide esters), US 5 118 678 (carbamates), US 5 100 883 (fluorinated esters), US 5 151 413 (acetals), and US 5 120 842 (silyl ethers), all of which are incorporated herein by reference. 32-O-dihydro or substituted rapamycin are described, e.g., in US 5 256 790, incorporated herein by reference.

It has now surprisingly been discovered that certain novel demethoxy derivatives of rapamycin (the Novel Compounds) have an improved pharmacological profile over rapamycin, exhibit greater stability and bioavailability, allow for greater ease in producing galenic formulations, and are more potent immunosuppressants. The Novel Compounds comprise rapamycins wherein the methoxy group(s) at position 16 and/or position 39 of rapamycin is deleted and replaced with a selected substituent. Without intending to be bound to any particular theory, we have hypothesized that these particular methoxy groups on rapamycin are targets for metabolic attack and can be replaced with particular selected substituents, optionally in combination with certain further modifications to the molecule, so that activity is retained, or even in some cases, enhanced, and at the same time, susceptibility to metabolic attack is reduced.

The Novel Compounds particularly include rapamycins (i) wherein the methoxy group at the 16 position is replaced with another substituent, preferably (optionally hydroxy-substituted) alkynyloxy, and/or (ii) wherein the methoxy group at the 39 position is deleted together with the 39 carbon so that the cyclohexyl ring of rapamycin

becomes a cyclopentyl ring lacking the 39 position methoxy group (i.e., 39-demethoxy-40-desoxy-39-substituted-42-nor-rapamycins, sometimes referred to herein simply as cyclopentyl rapamycins). The remainder of the molecule is as for rapamycin or its immunosuppressive derivatives and analogues, e.g., as described above. Optionally, the molecule is further modified, e.g., such that the hydroxy at the 40-position of rapamycin is alkylated, and/or the 32-carbonyl is reduced.

Preferably, the Novel Compounds are those having the structure of Formula I:



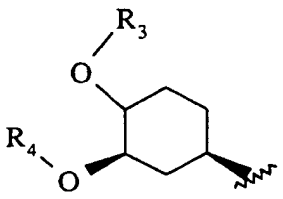
Formula I

wherein

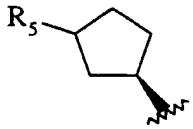
R_1 is selected from alkyl, alkenyl, alkynyl, hydroxyalkenyl, hydroxyalkyl, hydroxyalkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, alkoxyarylalkyl, haloalkyl, haloaryl, haloarylalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl; preferably an unsaturated substituent; more preferably an aromatic or alkynyl substituent; more

preferably alkynyl, hydroxyalkynyl, benzyl, alkoxybenzyl, or chlorobenzyl (wherein the substituted benzyl is *ortho*-substituted); most preferably alkynyl or hydroxyalkynyl;

R_2 is selected from formula II or formula III:



Formula II



Formula III

wherein

R_3 is selected from H, alkyl, alkenyl, alkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl; preferably hydroxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, alkoxyalkyl, and aminoalkyl; especially hydroxyethyl, hydroxypropyl, hydroxyethoxyethyl, methoxyethyl and acetylaminoethyl;

R_4 is H, methyl or together with R_3 forms C_{2-6} alkylene;

R_5 is substituted or unsubstituted acyl (e.g., formyl, carboxy, amide or ester), oxymethyl, iminomethyl, or dioxymethylyne (e.g., -O-CH-O-); preferably (i) oxymethyl, for example, hydroxymethyl, e.g., generally R_6O-CH_2-, wherein R_6 is selected from H, alkyl, alkenyl, alkynyl, aryl, amino, acyl (e.g., alkylcarbonyl, arylcarbonyl, heteroarylcarbonyl, hydroxyalkylcarbonyl, aminoalkylcarbonyl, or formyl), thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxyalkyl,

hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl; (ii) acyl, for example, (4-methyl-piperazin-1-yl)-carbonyl, (morpholin-4-yl)-carbonyl, or N-methyl-N-(2-pyridin-2-yl-ethyl)-carbamoyl, e.g., generally R_7CO-, wherein R_7 is selected from H, alkyl, hydroxy, alkoxy, aryloxy, amido, alkamido, a residue of an amino acid, or N,N-disubstituted-amido wherein the substituents (a) are selected from alkyl, aryl, arylalkyl or alkylaryl or (b) form a heterocyclic structure (e.g., morpholino or piperazino); (iii) iminomethyl, for example, p-toluenesulfonylhydrazonomethyl, e.g., generally R_8NCH-, wherein R_8 is alkyl, aryl, amino, alkylamino, arylamino, or arylsulfonylamino; or (iv) dioxysubstituted dioxymethylyne compounds, e.g., O,O-(alkylene)-dioxymethylyne (i.e., wherein the two oxygens are linked by an alkylene group); and

X and **Y** are independently selected from O, (H, OH), and (H, OR_9) wherein R_9 is selected from alkyl (preferably C_{1-4} alkyl), acyl (e.g., alkylcarbonyl, arylcarbonyl, heteroarylcarbonyl, hydroxyalkylcarbonyl, aminoalkylcarbonyl, or formyl), or aryl ;

wherein "alk" or "alkyl" refers to a C_{1-10} (preferably C_{1-6}) aliphatic substituent (branched, linear, or cyclic), optionally interrupted by an oxy (-O-) linkage; and "ar" or "aryl" refers to a monocyclic, optionally heterocyclic, optionally substituted, C_{4-14} aromatic substituent (e.g., tolyl, phenyl, benzyl, pyridyl, and the like);

provided that when R_2 is of formula II, then R_1 is other than methyl and (i) R_3 is selected from hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, and aminoalkyl; and/or (ii) X is other than O; and/or (iii) R_1 is (optionally hydroxy-substituted) alkynyl , preferably (optionally hydroxy-substituted) alk-2-ynyl, e.g. prop-2-ynyl, but-2-ynyl, pent-2-ynyl, or 4-hydroxy-but-2-ynyl;

and further provided that when R_1 is methyl, R_2 is of Formula III.

Demethoxy rapamycins of Formula I also include

(a) the 16-O substituted rapamycins wherein R_1 is selected from (i) benzyl, *ortho*-alkoxybenzyl, and chlorobenzyl (especially benzyl or *ortho*-methoxybenzyl), or (ii) (optionally hydroxy-substituted) alkynyl , preferably (optionally hydroxy-substituted) alk-

2-ynyl, especially (i) prop-2-ynyl, but-2-ynyl, pent-2-ynyl, and 4-hydroxy-but-2-ynyl; R_2 is of formula II; R_3 is selected from H, hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, and aminoalkyl; R_4 is methyl; and X and Y are independently selected from O, (H,OH), and (H, C_{1-4}alkoxy);

and most preferably, the 16-O substituted rapamycins wherein R_1 is alkynyl or hydroxyalkynyl, especially (optionally hydroxy substituted) C_{3-6} alk-2-ynyl; R_2 is of formula II; R_3 is selected from H, hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl; R_4 is methyl; and X and Y are O;

(b) the 16-O-substituted rapamycins wherein R_1 is selected from alkyl, alkyenyl, alkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl (especially alkynyl), wherein "alk" refers to C_{1-10} aliphatic substituent (branched, linear, or cyclic), optionally interrupted by an oxy (-O-) linkage, and aryl refers to a monocyclic aromatic substituent; provided that where R_1 is methyl, the compound is 16-epi-rapamycin; R_2 is of formula II; R_3 is H; R_4 is methyl; and X and Y are O; and

(c) the cyclopentyl rapamycins wherein R_2 is of Formula III, and R_1, R_5, X, and Y are as defined above; e.g., where R_1 is methyl, X and Y are O, and R_5 is substituted or unsubstituted acyl (e.g., formyl, carboxy, amide or ester), oxymethyl, iminomethyl, or dioxymethylyne (e.g., -O-CH-O-); e.g., (i) oxymethyl, e.g., R_6O-CH_2-, wherein R_6 is selected from H, alkyl, alkyenyl, alkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl; (ii) acyl, e.g., R_7CO-, wherein R_7 is selected from H, alkyl, hydroxy, alkoxy, aryloxy, amido, alkamido, a residue of an amino acid, or N,N-substituted-amido wherein the substituent forms a heterocyclic structure (e.g., morpholino or piperazino); (iii) iminomethyl, e.g., alkyliminomethyl, aryliminomethyl, or hydrazonomethyl; or (iv) dioxysubstituted

dioxymethylyne compounds, e.g., O,O-(alkylene)-dioxymethylyne (i.e., wherein the two oxygens are linked by an alkylene group); wherein "alk-" refers to a C_{1-6} aliphatic group (linear, branched, or cyclic) preferably C_{1-3}, in which the carbon chain may be optionally interrupted by an ether (-O-) linkage; and aryl refers to an aromatic group, preferably a monocyclic aromatic group.

Especially preferred compounds of Formula I include

1. 16-demethoxy-16-(pent-2-ynyl)oxy-rapamycin
2. 16-demethoxy-16-(but-2-ynyl)oxy-rapamycin
3. 16-demethoxy-16-(propargyl)oxy-rapamycin
4. 16-demethoxy-16-(4-hydroxy-but-2-ynyl)oxy-rapamycin
5. 16-demethoxy-16-benzyloxy-40-O-(2-hydroxyethyl)-rapamycin
6. 16-demethoxy-16-benzyloxy-rapamycin
7. 16-demethoxy-16-*ortho*-methoxybenzyl-rapamycin
8. 16-demethoxy-40-O-(2-methoxyethyl)-16-(pent-2-ynyl)oxy-rapamycin
9. 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin
10. 39-demethoxy-40-desoxy-39-hydroxymethyl-42-nor-rapamycin
11. 39-demethoxy-40-desoxy-39-carboxy-42-nor-rapamycin
12. 39-demethoxy-40-desoxy-39-(4-methyl-piperazin-1-yl)carbonyl-42-nor-rapamycin
13. 39-demethoxy-40-desoxy-39-(morpholin-4-yl)carbonyl-42-nor-rapamycin
14. 39-demethoxy-40-desoxy-39-[N-methyl, N-(2-pyridin-2-yl-ethyl)]carbamoyl-42-nor-rapamycin
15. 39-demethoxy-40-desoxy-39-(p-toluenesulfonylhydrazonomethyl)-42-nor-rapamycin

The compounds are produced from rapamycin or a rapamycin derivative generally as follows:

1. When the compound desired is of Formula I wherein R_1 is other than methyl, the modification at the 16-O can be produced either (i) by reaction of rapamycin or a rapamycin derivative with SeO_2 and a compound R_1-OH under suitable reaction conditions, e.g., at elevated temperatures, wherein R_1 is as defined above; or preferably (ii) by reaction of rapamycin or a rapamycin derivative with an acid, e.g., p-

toluenesulphonic acid, and a nucleophile, e.g., R_1-OH, at room temperature, in a suitable aprotic solvent, e.g., dichloromethane, acetonitrile, or THF.

2. When the compound desired is of formula I where R_2 is of formula II and R_3 is other than H, for example, O-alkylation at the C40 hydroxy is accomplished by reaction with an organic radical attached to a leaving group (e.g., R_3-Z where R_3 is an organic radical as defined above, e.g., an alkyl, allyl, or benzyl moiety, which is desired as the O-substituent, and Z is the leaving group, e.g., $CCl_3C(NH)O$ or CF_3SO_3) under suitable reaction conditions, e.g., in the presence of an acid like trifluoromethanesulfonic acid, camphorsulfonic acid, p-toluenesulfonic acid or their respective pyridinium or substituted pyridinium salts when Z is $CCl_3C(NH)O$ or in the presence of a base like pyridine, a substituted pyridine, diisopropylethylamine or pentamethylpiperidine when Z is CF_3SO_3, or analogously to the methods described in US 5 258 389 or PCT/EP 93/02604 for 40-O alkylation of rapamycin.

3. When the compound desired is of formula I where R_2 is of formula III, conversion of the cyclohexyl ring of formula II to the cyclopentyl ring of formula III is accomplished by reaction with morpholinosulphur trifloride to obtain the aldehyde compound (e.g., where R_5 is formyl). This compound thus obtained may then be oxidized from the aldehyde to the carboxylic acid (e.g., where R_5 is carboxy), or reduced from the aldehyde to the alcohol (e.g., where R_5 is hydroxymethyl). Further O-substitution or modification to make the other compounds of the invention is performed according to processes known to those skilled in the art, e.g., the following general processes: (i) for oxymethyl derivatives, the alcohol compound is reacted analogously as described above for 40-O-substitution; (ii) for acyl derivatives, the carboxylic acid compound is reacted with the desired amine or alcohol in the presence of an activating or coupling reagent, e.g., oxalylchloride or dicyclohexylcarbodiimide, to give the desired amide or ester compounds respectively; and (iii) for iminomethyl or dioxymethylyne compounds, the aldehyde compound is condensed with the desired amine or alkylenediol, respectively, under acidic conditions.

4. When the compound desired is of formula I where X is other than O, the 32-O-dihydro compound (where X is (H,OH)) is prepared by O-protecting the hydroxy groups, e.g., at positions 28 and 40 of rapamycin, e.g., using triethylsilyl ether protecting

groups, reducing the protected compound, e.g., using L-selectride, and optionally deprotecting, e.g., under mildly acidic conditions, analogously to the method described in US 5 256 790 for preparation of 32-O-dihydro-rapamycin from rapamycin. Where substitution at the 32 hydroxy is desired, the 28,40-O,O-protected compound is alkylated, e.g., as described for 40-O alkylation above, acylated, or otherwise O-substituted, e.g., analogously to the procedures described in US 5 256 790.

The above processes may be carried out in any order, preferably using rapamycin as the ultimate starting material. Where necessary, the starting materials and intermediates may be protected (e.g., O-protected as described in process 4) before carrying out the above reaction(s) and then deprotected to obtain the desired final product.

The Novel Compounds are particularly useful for the following conditions:

a) Treatment and prevention of organ or tissue transplant rejection, e.g. for the treatment of recipients of e.g. heart, lung, combined heart-lung, liver, kidney, pancreatic, skin or corneal transplants; including treatment and prevention of acute rejection; treatment and prevention of hyperacute rejection, e.g., as associated with xenograft rejection; and treatment and prevention of chronic rejection, e.g., as associated with graft-vessel disease. The Novel Compounds are also indicated for the treatment and prevention of graft-versus-host disease, such as following bone marrow transplantation.

b) Treatment and prevention of autoimmune disease and of inflammatory conditions, in particular inflammatory conditions with an etiology including an autoimmune component such as arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans) and rheumatic diseases. Specific autoimmune diseases for which the compounds of the invention may be employed include, autoimmune hematological disorders (including e.g. hemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, sclerodoma, Wegener granulamatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's

disease) endocrine ophthalmopathy, Graves disease, sarcoidosis, multiple sclerosis, primary billiary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis, glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy) and juvenile dermatomyositis.

c) Treatment and prevention of asthma.

d) Treatment of multi-drug resistance (MDR). The Novel Compounds suppress P-glycoproteins (Pgp), which are the membrane transport molecules associated with MDR. MDR is particularly problematic in cancer patients and AIDS patients who will not respond to conventional chemotherapy because the medication is pumped out of the cells by Pgp. The Novel Compounds are therefore useful for enhancing the efficacy of other chemotherapeutic agents in the treatment and control of multidrug resistant conditions such as multidrug resistant cancer or multidrug resistant AIDS.

e) Treatment of proliferative disorders, e.g. tumors, hyperproliferative skin disorder and the like.

f) Treatment of fungal infections.

g) Treatment and prevention of inflammation, especially in potentiating the action of steroids.

h) Treatment and prevention of infection, especially infection by pathogens having Mip or Mip-like factors.

The invention thus provides the Novel Compounds described herein, for use as novel intermediates or as pharmaceuticals, methods of treating or preventing the above-described disorders by administering an effective amount of a Novel Compound to a patient in need thereof, use of a Novel Compound in the manufacture of a medicament for treatment or prevention of the above-described disorders, and pharmaceutical compositions comprising a Novel Compound in combination or association with a pharmaceutically acceptable diluent or carrier.

The Novel Compounds are utilized by administration of a pharmaceutically effective dose in pharmaceutically acceptable form to a subject in need of treatment. Appropriate dosages of the Novel Compounds will of course vary, e.g. depending on the condition to be treated (for example the disease type or the nature of resistance), the effect desired and the mode of administration.

In general however satisfactory results are obtained on administration orally at dosages on the order of from 0.05 to 5 or up to 10mg/kg/day, e.g. on the order of from 0.1 to 2 or up to 7.5 mg/kg/day administered once or, in divided doses 2 to 4x per day, or on administration parenterally, e.g. intravenously, for example by i.v. drip or infusion, at dosages on the order of from 0.01 to 2.5 up to 5 mg/kg/day, e.g. on the order of from 0.05 or 0.1 up to 1.0 mg/kg/day. Suitable daily dosages for patients are thus on the order of 500 mg p.o., e.g. on the order of from 5 to 100 mg p.o., or on the order of from 0.5 to 125 up to 250 mg i.v., e.g. on the order of from 2.5 to 50 mg i.v..

Alternatively and even preferably, dosaging is arranged in patient specific manner to provide pre-determined trough blood levels, e.g. as determined by RIA technique. Thus patient dosaging may be adjusted so as to achieve regular on-going trough blood levels as measured by RIA on the order of from 50 or 150 up to 500 or 1000ng/ml, i.e. analogously to methods of dosaging currently employed for Ciclosporin immunosuppressive therapy.

The Novel Compounds may be administered as the sole active ingredient or together with other drugs. For example, in immunosuppressive applications such as prevention and treatment of graft vs. host disease, transplant rejection, or autoimmune disease, the Novel Compounds may be used in combination with cyclosporins or ascomycins, or their immunosuppressive analogs, e.g., cyclosporin A, cyclosporin G, FK-506, etc.; corticosteroids; cyclophosphamide; azathioprene; methotrexate; brequinar; leflunomide; mizoribine; immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., MHC, CD2, CD3, CD4, CD7, CD25, CD28, CTLA4, B7, CD45, or CD58 or their ligands; or other immunomodulatory compounds.

For immunosuppressive applications, e.g., treatment and prevention of organ or tissue transplant rejection, the combination is most preferably with IL-2 transcription inhibitors such as the immunosuppressive cyclosprins (e.g., cyclosporin A) and ascomycins (e.g., FK-506). For anti-inflammatory applications, the Novel Compounds can also be used together with anti-inflammatory agents, e.g., corticosteroids. For anti-infective applications, the Novel Compounds can be used in combination with other anti-infective agents, e.g., anti-viral drugs or antibiotics.

The Novel Compounds are administered by any conventional route, in particular enterally, e.g. orally, for example in the form of solutions for drinking, tablets or capsules or parenterally, for example in the form of injectable solutions or suspensions. Suitable unit dosage forms for oral administration comprise, e.g. from 1 to 50 mg of a compound of the invention, usually 1 to 10 mg. Pharmaceutical compositions comprising the novel compounds may be prepared analogously to pharmaceutical compositions comprising rapamycin, e.g., as described in EPA 0 041 795, which would be evident to one skilled in the art.

The pharmacological activities of the Novel Compounds are demonstrated in, e.g., the following tests:

1. Mixed lymphocyte reaction (MLR)

The Mixed Lymphocyte Reaction was originally developed in connection with allografts, to assess the tissue compatibility between potential organ donors and recipients, and is one of the best established models of immune reaction in vitro. A murine model MLR, e.g., as described by T.Meo in "Immunological Methods", L. Lefkovits and B. Peris, Eds., Academic Press, N.Y. pp. 227-239 (1979), is used to demonstrate the immunosuppressive effect of the Novel Compounds. Spleen cells (0.5×10^6) from Balb/c mice (female, 8-10 weeks) are co-incubated for 5 days with 0.5×10^6 irradiated (2000 rads) or mitomycin C treated spleen cells from CBA mice (female, 8-10 weeks). The irradiated allogeneic cells induce a proliferative response in the Balb/c spleen cells which can be measured by labeled precursor incorporation into the DNA.

Since the stimulator cells are irradiated (or mitomycin C treated) they do not respond to the Balb/c cells with proliferation but do retain their antigenicity. The antiproliferative effect of the Novel Compounds on the Balb/c cells is measured at various dilutions and the concentration resulting in 50% inhibition of cell proliferation (IC_{50}) is calculated. All of the exemplified Novel Compounds are active in this assay. The alkynyl derivatives of the examples are particularly potent immunosuppressants, with an IC_{50} in this assay relative to rapamycin of 0.3 - 0.8, i.e., up to 3x more active than rapamycin.

2. IL-6 mediated proliferation

The capacity of the Novel Compounds to interfere with growth factor associated signalling pathways is assessed using an interleukin-6 (IL-6)-dependent mouse hybridoma cell line. The assay is performed in 96-well microtiter plates. 5000 cells/well are cultivated in serum-free medium (as described by M. H. Schreier and R. Tees in Immunological Methods, I. Lefkovits and B. Pernis, eds., Academic Press 1981, Vol. II, pp. 263-275), supplemented with 1 ng recombinant IL-6/ml. Following a 66 hour incubation in the absence or presence of a test sample, cells are pulsed with 1 μCi (3-H)-thymidine/well for another 6 hours, harvested and counted by liquid scintillation. (3-H)-thymidine incorporation into DNA correlates with the increase in cell number and is thus a measure of cell proliferation. A dilution series of the test sample allows the calculation of the concentration resulting in 50% inhibition of cell proliferation (IC_{50}). All of the exemplified Novel Compounds are active in this assay. The alkynyl derivatives of the examples are particularly potent immunosuppressants, with an IC_{50} in this assay relative to rapamycin of from 0.2 to 0.9, i.e., up to 5x more active than rapamycin.

3. Macrophilin binding assay

Rapamycin and the structurally related immunosuppressant, FK-506, are both known to bind in vivo to macrophilin-12 (also known as FK-506 binding protein or FKBP-12), and this binding is thought to be related to the immunosuppressive activity of these compounds. The Novel Compounds also bind strongly to macrophilin-12, as is demonstrated in a competitive binding assay. In this assay, FK-506 coupled to BSA is

used to coat microtiter wells. Biotinylated recombinant human macrophilin-12 (biot-MAP) is allowed to bind in the presence or absence of a test sample to the immobilized FK-506. After washing (to remove non-specifically bound macrophilin), bound biot-MAP is assessed by incubation with a streptavidin-alkaline phosphatase conjugate, followed by washing and subsequent addition of p-nitrophenyl phosphate as a substrate. The read-out is the OD at 405nm. Binding of a test sample to biot-MAP results in a decrease in the amount of biot-MAP bound to the FK-506 and thus in a decrease in the OD405. A dilution series of the test sample allows determination of the concentration resulting in 50% inhibition of the biot-MAP binding to the immobilized FK-506 (IC_{50}). The exemplified Novel Compounds all exhibit good binding to FKBP in this assay.

4. Localized Graft-Versus-Host (GvH) Reaction

In vivo efficacy of the Novel Compounds is proved in a suitable animal model, as described, e.g., in Ford et al, TRANSPLANTATION 10 (1970) 258. Spleen cells (1 x 10^7) from 6 week old female Wistar/Furth (WF) rats are injected subcutaneously on day 0 into the left hind-paw of female (F344 x WF)F_1 rats weighing about 100g. Animals are treated for 4 consecutive days and the popliteal lymph nodes are removed and weighed on day 7. The difference in weight between the two lymph nodes is taken as the parameter for evaluating the reaction.

5. Kidney Allograft Reaction in Rat

One kidney from a female fisher 344 rat is transplanted onto the renal vessel of a unilaterally (left side) nephrectomized WF recipient rat using an end-to-end anastomosis. Ureteric anastomosis is also end-to-end. Treatment commences on the day of transplantation and is continued for 14 days. A contralateral nephrectomy is done seven days after transplantation, leaving the recipient relying on the performance of the donor kidney. Survival of the graft recipient is taken as the parameter for a functional graft.

6. Experimentally Induced Allergic Encephalomyelitis (EAE) in Rats

Efficacy of the Novel Compounds in EAE is measured, e.g., by the procedure described in Levine & Wenk, AMER J PATH 47 (1965) 61; McFarlin et al, J IMMUNOL 113 (1974) 712; Borel, TRANSPLANT. & CLIN. IMMUNOL 13 (1981) 3. EAE is a widely accepted model for multiple sclerosis. Male Wistar rats are injected in the hind paws with a mixture of bovine spinal cord and complete Freund's adjuvant. Symptoms of the disease (paralysis of the tail and both hind legs) usually develop within 16 days. The number of diseased animals as well as the time of onset of the disease are recorded.

7. Freund's Adjuvant Arthritis

Efficacy against experimentally induced arthritis is shown using the procedure described, e.g., in Winter & Nuss, ARTHRITIS & RHEUMATISM 9 (1966) 394; Billingham & Davies, HANDBOOK OF EXPERIMENTAL PHARMACOL (Vane & Ferreira Eds, Springer-Verlag, Berlin) 50/II (1979) 108-144. OFA and Wistar rats (male or female, 150g body weight) are injected i.c. at the base of the tail or in the hind paw with 0.1 ml of mineral oil containing 0.6 mg of lyophilized heat-killed Mycobacterium smegmatis. In the developing arthritis model, treatment is started immediately after the injection of the adjuvant (days 1 - 18); in the established arthritis model treatment is started on day 14, when the secondary inflammation is well developed (days 14-20). At the end of the experiment, the swelling of the joints is measured by means of a micro-caliper. ED_{50} is the oral dose in mg/kg which reduces the swelling (primary or secondary) to half of that of the controls.

8. Antitumor and MDR activity

The antitumor activity of the Novel Compounds and their ability to enhance the performance of antitumor agents by alleviating multidrug resistance is demonstrated, e.g., by administration of an anticancer agent, e.g., colchicine or etoposide, to multidrug resistant cells and drug sensitive cells in vitro or to animals having multidrug resistant or drug sensitive tumors or infections, with and without co-administration of the Novel Compounds to be tested, and by administration of the Novel Compound alone. Such in vitro testing is performed employing any appropriate drug resistant cell line and control

(parental) cell line, generated, e.g. as described by Ling et al., J. Cell. Physiol. 83, 103-116 (1974) and Bech-Hansen et al. J. Cell. Physiol. 88, 23-32 (1976). Particular clones chosen are the multi-drug resistant (e.g. colchicine resistant) line CHR (subclone C5S3.2) and the parental, sensitive line AUX B1 (subclone AB1 S11). In vivo anti-tumor and anti-MDR activity is shown, e.g., in mice injected with multidrug resistant and drug sensitive cancer cells. Ehrlich ascites carcinoma (EA) sub-lines resistant to drug substance DR, VC, AM, ET, TE or CC are developed by sequential transfer of EA cells to subsequent generations of BALB/c host mice in accordance with the methods described by Slater et al., J. Clin. Invest, 70, 1131 (1982). Equivalent results may be obtained employing the Novel Compounds test models of comparable design, e.g. in vitro, or employing test animals infected with drug-resistant and drug sensitive viral strains, antibiotic (e.g. penicillin) resistant and sensitive bacterial strains, anti-mycotic resistant and sensitive fungal strains as well as drug resistant protozoal strains, e.g. Plasmodial strains, for example naturally occurring sub-strains of Plasmodium falciparum exhibiting acquired chemotherapeutic, anti-malarial drug resistance.

9. Steroid potentiation

The macrophilin binding activity of the Novel Compounds also makes them useful in enhancing or potentiating the action of corticosteroids. Combined treatment with the compounds of the invention and a corticosteroid, such as dexamethasone, results in greatly enhanced steroidal activity. This can be shown, e.g., in the murine mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter gene assay, e.g., as described in Ning, et al., *J. Biol. Chem.* (1993) **268**: 6073. This synergistic effect allows reduced doses of corticosteroids, thereby reducing the risk of side effects in some cases.

10. Mip and Mip-like factor inhibition

Additionally, the Novel Compounds bind to and block a variety of Mip (macrophage infectivity potentiator) and Mip-like factors, which are structurally similar to macrophilin. Mip and Mip-like factors are virulence factors produced by a wide variety of pathogens, including those of the genera Chlamidia, e.g., Chlamidia

trachomatis; Neisseria, e.g., Neisseria meningitidis; and Legionella, e.g., Legionella pneumophilia; and also by the obligately parasitic members of the order Rickettsiales. These factors play a critical role in the establishment of intracellular infection. The efficacy of the Novel Compounds in reducing the infectivity of pathogens which produce Mip or Mip-like factors can be shown by comparing infectivity of the pathogens in cells culture in the presence and absence of the macrolides, e.g., using the methods described in Lundemose, et al., *Mol. Microbiol.* (1993) 7: 777.

The Novel Compounds are also useful in assays to detect the presence or amount of macrophilin-binding compounds, e.g., in competitive assays for diagnostic or screening purposes. Thus, in another embodiment, the invention provides for use of the Novel Compounds as a screening tool to determine the presence of macrophilin-binding compounds in a test solution, e.g., blood, blood serum, or test broth to be screened. Preferably, a Novel Compound is immobilized in microtiter wells and then allowed to bind in the presence and absence of a test solution to labelled macrophilin-12 (FKBP-12). Alternatively, the FKBP-12 immobilized in microtiter wells and allowed to bind in the presence and absence of a test solution to a Novel Compound which has been labelled, e.g., fluoro-, enzymatically- or radio-labelled, e.g., a Novel Compound of Formula I wherein R_1 comprises a labelling group. The plates are washed and the amount of bound labelled compound is measured. The amount of macrophilin-binding substance in the test solution is roughly inversely proportional to the amount of bound labelled compound. For quantitative analysis, a standard binding curve is made using known concentrations of macrophilin binding compound.

The following examples are intended to illustrate rather than limit the invention. Characteristic spectrascopic data is provided to aid in identification of the compounds.

Example 1: 16-demethoxy-16-(pent-2-ynyl)oxy-rapamycin

To a solution of 0.6 ml 2-pentyn-1-ol in 5 ml CH_2Cl_2 are added 456 mg rapamycin followed by 5 mg p-toluenesulfonic acid. The mixture is stirred for 2 h at room temperature. Then the reaction is quenched with 7 ml of a saturated aqueous solution of

$NaHCO_3$. The aqueous phase is separated and extracted 2x with 10 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl acetate/hexane 3/2. The crude product is finally purified by preparative HPLC (RP-18, 250x10 mm, MeOH/H2O 80/20, 3 ml/min).

MS (FAB) m/z 972 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.67 (1H, q); 1.13 (3H, t); 1.67 (3H,s); 1.74 (3H, s); 3.33 (3H, s); 3.40 (3H, s); 3.73 (1H, d); 3.77 (1H, dm); 4.01 (1H, dm); 4.16 (1H, d); 4.66 (1H, s).

Example 2: 16-demethoxy-16-(but-2-ynyl)oxy-rapamycin

To a solution of 0.4 ml 2-butyn-1-ol in 3 ml CH_2Cl_2 are added 251 mg rapamycin followed by 4 mg p-toluenesulfonic acid. The mixture is stirred for 2 h at room temperature. Then the reaction is quenched with 7 ml of a saturated aqueous solution of NaHCO3. The aqueous phase is separated and extracted 2x with 10 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl acetate/hexane 3/2. The crude product is finally purified by preparative HPLC (RP-18, 250x10 mm, MeOH/H2O 80/20, 3 ml/min).

MS (FAB) m/z 958 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.67 (1H, q); 1.67 (3H,s); 1.74 (3H, s); 1.83 (1H, bs); 3.33 (3H, s); 3.40 (3H, s); 3.72 (1H, d); 3.75 (1H, dm); 4.01 (1H, dm); 4.16 (1H, d); 4.73 (1H, s).

Example 3: 16-demethoxy-16-(propargyl)oxy-rapamycin

To a solution of 0.3 ml propargyl alcohol in 3 ml CH_2Cl_2 are added 251 mg rapamycin followed by 4 mg p-toluenesulfonic acid. The mixture is stirred for 2 h at room temperature. Then the reaction is quenched with 7 ml of a saturated aqueous solution of NaHCO3. The aqueous phase is separated and extracted 2x with 10 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl

acetate/hexane 3/2. The crude product is finally purified by preparative HPLC (RP-18, 250x10 mm, MeOH/H2O 80/20, 3 ml/min).

MS (FAB) m/z 944 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.68 (1H, q); 1.66 (3H,s); 1.74 (3H, s); 2.32 (1H, bt); 3.34 (3H, s); 3.41 (3H, s); 3.67 (1H, d); 3.83 (1H, dm); 4.08 (1H, dm); 4.16 (1H, d); 4.84 (1H, s).

Example 4: 16-demethoxy-16-(4-hydroxy-but-2-ynyl)oxy-rapamycin

To a suspension of 940 mg 2-butyn-1,4-diol in 6 ml CH_2Cl_2 are added 502 mg rapamycin followed by 5 mg p-toluenesulfonic acid. The mixture is stirred for 2 h at room temperature. Then the reaction is quenched with 10 ml of a saturated aqueous solution of $NaHCO_3$. The aqueous phase is separated and extracted 2x with 10 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl acetate/hexane 4/1. The crude product is finally purified by preparative HPLC (RP-18, 250x25 mm, MeOH/H2O 75/25, 7 ml/min).

MS (FAB) m/z 974 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.67 (1H, q); 1.67 (3H,s); 1.75 (3H, s); 3.33 (3H, s); 3.41 (3H, s); 3.73 (1H, d); 3.81 (1H, dm); 4.08 (1H, dm); 4.17 (1H, d); 4.28 (2H, bs); 4.67 (1H, s).

Example 5: 16-demethoxy-16-benzyloxy-40-O-(2-hydroxyethyl)-rapamycin

To a solution of 0.6 ml benzyl alcohol in 3 ml CH_2Cl_2 are added 264 mg 40-O-(2-hydroxyethyl)-rapamycin (prepared as described in WO 94/09010) followed by 5 mg p-toluenesulfonic acid. The mixture is stirred for 1 h at room temperature. Then the reaction is quenched with 7 ml of a saturated aqueous solution of $NaHCO_3$. The aqueous phase is separated and extracted 2x with 10 ml diethyl ether. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl hexane/acetone 4/1 followed by hexane/acetone 1/1. The crude product is finally purified by preparative HPLC (RP-18, 250x25 mm, CH3CN/H2O 75/25, 8 ml/min).

MS (FAB) m/z 1040 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.72 (1H, q); 1.73 (6H,s); 3.32 (3H, s); 3.43 (3H, s); 3.7 (4H, m); 4.15 (1H, d); 4.18 (1H, d); 4.47 (1H, d)); 4.80 (1H, s); 7.3 (5H, m).

Example 6: 16-demethoxy-16-benzyloxy-rapamycin:

1 mmol rapamycin is dissolved in 50 ml methylene chloride containing 3 ml of benzyl alcohol. 0.1 mmol of p-toluenesulphonic acid is added, and the reaction mixture is then stirred at room temperature for 2-10 hours. The reaction mixture is then poured in a saturated solution of sodium bicarbonate. The organic layer is separated, dried over sodium sulphate, and the solvent evaporated. The crude product is then purified by HPLC to give the pure title compound as a white powder.

Example 7: 16-demethoxy-16-(*ortho*-methoxybenzyl)oxy-rapamycin

To a solution of 0.76 g of *ortho*-methoxy-benzyl alcohol in 3 mL CH_2Cl_2 are added 250 mg of rapamycin followed by 5 mg of p-toluenesulfonic acid. The mixture is stirred for 8 h at room temperature and the reaction is quenched with 5 mL of a saturated aqueous solution of $NaHCO_3$. The layers are separated and the aqueous layer is extracted 2x with 10 mL ether. The combined organic solution is dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue is chromatographed over silica gel, using hexane/acetone (4/1 to 3/2) as the eluent. The resulting product is further purified by preparative HPLC (RP-18, 250x25 mm, CH_3CN/H_2O 75/25, 8 mL/min).

MS (FAB) m/z 1026 (M+Li)

H-NMR ($CDCl_3$) (major isomer) δ: 0.67 (1H, q); 1.73 and 1.74 (6H, 2s); 3.33 (3H, s); 3.41 (3H, s); 3.72 (1H, d); 3.81 (3H, s); 4.18 (1H, broad d); 4.26 (1H, d); 4.45 (1H, d); 4.72 (1H, broad s); 6.83 (1H, d); 6.92 (1H, m); 7.23 (1H, m); 7.32 (1H, m).

Example 8: 16-demethoxy-40-O-(2-methoxyethyl)-16-(pent-2-ynyl)oxy-rapamycin

To a solution of 0.7 ml 2-pentyn-1-ol in 5 ml CH_2Cl_2 are added 486 mg of 40-O-(2-methoxyethyl)-rapamycin followed by 5 mg p-toluenesulfonic acid. The mixture is stirred for 2 h at room temperature. Then the reaction is quenched

with 7 ml of a saturated aqueous solution of $NaHCO_3$. The aqueous phase is separated and extracted 2x with 10 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The residue is chromatographed over silica gel, eluting with ethyl acetate/hexane 1/1. The crude product is finally purified by preparative HPLC (RP-18, 250x25 mm, MeOH/H2O 83/17, 7 ml/min).

MS (FAB) m/z 1030 (M+Li)

H-NMR (CDCl3)(major isomer) d: 0.72 (1H, q); 1.14 (3H, t); 1.67 (3H,s); 1.74 (3H, s); 3.33 (3H, s); 3.38 (3H, s); 3.45 (3H, s); 3.73 (1H, d); 3.77 (1H, dm); 4.01 (1H, dm); 4.17 (1H, d); 4.65 (1H, s).

Example 9: 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin

To a solution of 1.85 g of rapamycin in 40 ml acetonitrile at -30 C are added 365 µl morpholinosulphur trifluoride. The reaction mixture is kept 1h at -30 C, 1h at 0 C and then quenched with a saturated aqueous bicarbonate solution. The aqueous phase is extracted 3x with 30 ml ethyl acetate, and the organic phases are combined and dried over sodium sulfate. After evaporation of the solvent, the crude product is purified by column chromatography over silica gel, eluting with hexane/acetone 4/1.

MS (FAB, LiI matrix) : 888 (M+ Li)

H-NMR (CDCl3): 3.13 (s, 3H); 3.34 (s, 3H); 9.62 (d, 1H); no other singulet between 3.0 and 3.6 ppm. No signal between 0.6 and 0.85 ppm

Example 10: 39-demethoxy-40-desoxy-39-hydroxymethyl-42-nor-rapamycin

A solution of 44 mg 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin in 1.2 ml of THF/water 5/1 is treated with 1.5 mg of t-butylamine/borane complex for 2h at 0 C. the reaction mixture is then poured on 2 ml HCl 0.1N and extracted with 3x 5 ml ethyl acetate. The organic phases are combined, washed with 2 ml of a saturated sodium bicarbonate solution and dried over sodium sulfate. The solvent is evaporated in vacuo, and the crude product is purified by column chromatography over silica gel eluting with hexane/ethyl acetate 1/1.

MS (FAB, LiI matrix): 890 (M + Li)

H-NMR (CDCl3): 3.13 (s, 3H); 3.33 (s, 3H); 4.18 (m, 2H). No signal betwwen 0.5 and 0.85 ppm.; no aldehyde proton at 9.62 ppm.

Example 11: 39-demethoxy-40-desoxy-39-carboxy-42-nor-rapamycin

A solution of 85 mg NaOCl and 113 mg NaH_2PO_4 in 2 ml water is added to a solution of 111 mg 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin and 0.2 ml 2-methyl-2-butene in 4 ml t-butanol. The mixture is stirred at room temperature for 2h. The solvents are then evaporated and the residue extracted with 3x 5 ml ethyl acetate. The organic phases are combined, dried over anhydrous sodium sulfate and the solvent evaporated. The product is purified by preparative HPLC (RP-18, 250x10 mm, acetonitrile/water 60/40, 3 ml/mn).

MS (FAB, LiI matrix): 904 (M+Li)

H-NMR(CDCl3): 1.65(s, 3H); 1.78(s, 3H); 3.13(s, 3H); 3.33(s, 3H); 3.75(d, 1H); 4.18 (d, 1H). No signal below 0.85 ppm. No additional singulet in the region 3.0-3.6 ppm.

Example 12:

39-demethoxy-40-desoxy-39-(4-methyl-piperazin-1-yl)carbonyl-42-nor-rapamycin

To a stirred solution of 180 mg 39-carboxy-39-demethoxy-40-desoxy-42-nor-rapamycin in 4 ml THF at - 75 C are added 0.08 ml pyridine followed by 0.04 ml oxalyl chloride. The reaction mixture is kept at - 75 C for 30 minutes after which 0.09 ml N-methyl-piperazine are added. The reaction is stirred for an additional hour and then quenched with 5 ml of saturated aqueous sodium bicarbonate and 5 ml ethyl acetate. The water phase is separated and extracted with 2x 5 ml ethyl acetate. The organic phases are combined, dried over sodium sulfate and the solvent evaporated. The crude product is purified by preparative HPLC (RP-18, 250x10 mm, MeOH/H2O 85/15, 3 ml/mn).

MS (FAB) m/z 986 (M+Li)

H-NMR (CDCl3) d= 1.65 (3H, s); 1.78 (3H, s); 2.31 (3H, s); 2.4 (4H, m); 3.13 (3H, s); 3.34 (3H, s); 3.79 (1H, d); 4.21 (1H, d); 4.68 (1H, bs).

Example 13: 39-demethoxy-40-desoxy-39-(morpholin-4-yl)carbonyl-42-nor-rapamycin

This compound is obtained following the method of Example 11, using morpholine instead of N-methyl-piperazine.

MS (FAB) m/z 973 (M+Li)

H-NMR (CDCl3) d= 1.65 (3H, s); 1.77 (3H, s); 3.13 (3H, s); 3.33 (3H, s); 3.6 (4H, m); 3. 77 (1H, d); 4.19 (1H, d); 4.66 (1H, bs).

Example 14: 39-demethoxy-40-desoxy-39-[N-methyl,N-(2-pyridin-2-yl-ethyl)]carbamoyl-42-nor-rapamycin

This compound is obtained following the method of Example 11 using (2-pyridin-2-yl-ethyl)methylamine instead of N-methyl-piperazine.

MS (FAB) m/z 1022 (M+Li)

H-NMR (CDCl3) d= 1.66 (3H, s); 1.78 (3H, s); 2.93 (3H, s); 3.13 (3H, s); 3.33 (3H, s); 4.23 (1H, m); 4.67 (1H, s); 7.1 (2H, m); 7.6 (1H, m); 8.51 (1H, d).

Example 15:

39-demethoxy-40-desoxy-39-(p-toluenesulfonylhydrazonomethyl)-42-nor-rapamycin

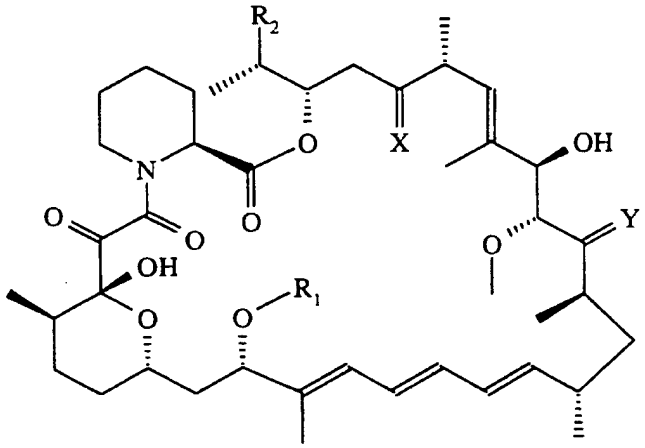
To a mixture of 523 mg 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin in 10 ml acetonitrile are added 156 mg p-toluenesulfonylhydrazide. The reaction mixture is stirred for 30 minutes at room temperature and then the solvent is evaporated. The residue is chromatographed over silica gel, eluting with hexane/acetone 5/1, to give the title compound.

MS (FAB) m/z 1056 (M+Li)

H-NMR (CDCl3) d= 1.65 (3H, s); 1.76 (3H, s); 2.43 (3H, s); 3.13 (3H, s); 3.34 (3H, s); 3.79 (1H, d); 4.18 (1H, d); 4.69 (1H, bs); 7.13 (1H, d); 7.32 (2H, d); 7.56 (1H, s); 7.80 (2H, d).

CLAIMS

1. A compound of Formula I:

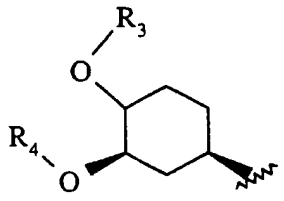


Formula I

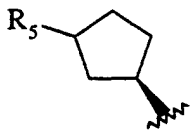
wherein

R_1 is selected from alkyl, alkenyl, alkynyl, hydroxyalkyl, hydroxyalkenyl, hydroxyalkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, alkoxyarylalkyl, haloalkyl, haloaryl. haloarylalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl;

R_2 is selected from formula II or formula III:



Formula II



Formula III

wherein

R_3 is selected from H, alkyl, alkyenyl, alkynyl, aryl, thioalkyl, arylalkyl, hydroxyarylalkyl, hydroxyaryl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxyalkyl, hydroxyalkylarylalkyl, dihydroxyalkylarylalkyl, alkoxyalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylamidoalkyl, acylamidoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, carbalkoxyalkyl, and alkylsilyl;

R_4 is H, methyl or together with R_3 forms C_{2-6} alkylene;

R_5 is substituted or unsubstituted acyl, oxymethyl, iminomethyl, or dioxymethylyne;

wherein "alk" or "alkyl" refers to a C_{1-10} aliphatic substituent (branched, linear, or cyclic), optionally interrupted by an oxy (-O-) linkage; and "ar" or "aryl" refers to a monocyclic, optionally heterocyclic, optionally substituted, C_{4-14} aromatic substituent;

provided that when R_2 is of formula II, then R_1 is other than methyl and (i) R_3 is selected from hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, and aminoalkyl; and/or (ii) X is other than O; and/or (iii) R_1 is (optionally hydroxy-substituted) alkynyl;

and further provided that when R_1 is methyl, R_2 is of Formula III.

2. A compound according to claim 1 of Formula I wherein R_1 is selected from benzyl, *ortho*-alkoxybenzyl, chlorobenzyl, and (optionally hydroxy-substituted) alkynyl; R_2 is of formula II; R_3 is selected from H, hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, and aminoalkyl; R_4 is methyl; and X and Y are independently selected from O, (H,OH), and (H, C_{1-4}alkoxy);

3. A compound according to claim 2 of Formula I wherein R_1 is (optionally hydroxy-substituted) C_{3-6} alk-2-ynyl; R_2 is of formula II; R_3 is selected from H, hydroxyalkyl, alkoxyalkyl, hydroxyalkoxyalkyl; R_4 is methyl; and X and Y are O;

4. A compound according to claim 1 of Formula I wherein R_2 is of Formula III.

5. A compound according to claim 1 selected from

i. 16-demethoxy-16-(pent-2-ynyl)oxy-rapamycin
ii. 16-demethoxy-16-(but-2-ynyl)oxy-rapamycin
iii. 16-demethoxy-16-(propargyl)oxy-rapamycin
iv. 16-demethoxy-16-(4-hydroxy-but-2-ynyl)oxy-rapamycin
v. 16-demethoxy-16-benzyloxy-40-O-(2-hydroxyethyl)-rapamycin
vi. 16-demethoxy-16-benzyloxy-rapamycin
vii. 16-demethoxy-16-*ortho*-methoxybenzyl-rapamycin
viii. 16-demethoxy-40-O-(2-methoxyethyl)-16-(pent-2-ynyl)oxy-rapamycin
ix. 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin
x. 39-demethoxy-40-desoxy-39-hydroxymethyl-42-nor-rapamycin
xi. 39-demethoxy-40-desoxy-39-carboxy-42-nor-rapamycin
xii. 39-demethoxy-40-desoxy-39-(4-methyl-piperazine-1-carbonyl)-42-nor-rapamycin
xiii. 39-demethoxy-40-desoxy-39-(morpholin-4-yl)carbonyl-42-nor-rapamycin
xiv. 39-demethoxy-40-desoxy-39-[N-methyl,N-(2-pyridin-2-yl-ethyl)]carbamoyl-42-nor-rapamycin
xv. 39-demethoxy-40-desoxy-39-(p-toluenesulfonylhydrazonomethyl)-42-nor-rapamycin

6. A compound according to any one of claims 1 through 5 for use as a pharmaceutical.

7. A pharmaceutical composition comprising a compound according to any one of claims 1 through 4 together with a pharmaceutically acceptable diluent or carrier.

8. Use of a compound according to any one of claims 1 through 4 in the manufacture of a medicament for treating or preventing any of the following conditions:

(i) autoimmune disease,
(ii) acute rejection of organ or tissue transplant,
(iii) hyperacute rejection of organ or tissue transplant,
(iii) chronic rejection of organ or tissue transplant,
(iii) graft vs. host disease,
(iv) asthma,
(v) multidrug resistance,
(vi) tumors or hyperproliferative disorders, or
(vii) fungal infections,
(viii) inflammation, or
(ix) infection by pathogens having Mip or Mip-like factors.

9. A process for making a compound of Formula I comprising one or more of the following steps:

i. When the compound desired is for Formula I wherein R_1 is other than methyl, reacting rapamycin or a derivative thereof with SeO_2 and a compound R_1-OH under suitable reaction conditions, wherein R_1 is as defined as for Formula I, or reacting rapamycin or a derivative thereof with an acid and a compound R_1-OH in a suitable aprotic solvent;

ii. When the compound desired is of formula I where R_2 is of formula II and R_3 is other than H, reacting rapamycin or a derivative thereof with an organic radical attached to a leaving group R_3-Z where R_3 is an organic radical as defined in Formula I which is desired as the O-substituent, and Z is the leaving group (preferably $CCl_3C(NH)O$ or CF_3SO_3) in the presence of a suitable acid, e.g., when Z is $CCl_3C(NH)O$, or in the presence of a suitable base, e.g., when Z is CF_3SO_3;

iii. When the compound desired is of formula I where R_2 is of formula III, reacting rapamycin or a derivative thereof with morpholinosulphur trifloride to obtain the aldehyde compound, then optionally oxidizing the aldehyde to the carboxylic acid or

reducing the aldehyde to the corresponding alcohol; and further optionally (a) O-substituting the alcohol thus obtained, as in step ii, or (b) reacting the carboxylic acid thus obtained with an amine or alcohol in the presence of an activating or coupling reagent to give the desired amide or ester compounds respectively, or (c) condensing the aldehyde thus obtained with the desired amine or alkylenediol, respectively, under acidic conditions to obtain the iminomethyl or dioxymethylyne compounds respectively;

iv. When the compound desired is of formula I where X is other than O, reducing a rapamycin or derivative (in O-protected form) at the 32-keto to obtain the alcohol and optionally further O-substituting as in step ii;

v. Optionally protecting and deprotecting as necessary;

and recovering the compound of Formula I thus obtained.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 94/04191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D498/18 A61K31/395 C07F7/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07D A61K C07F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US,A,5 310 903 (GOULET) 10 May 1994 see claim 1	1-9
P,X	US,A,5 310 901 (PARSONS) 10 May 1994 see claim 1	1-9
P,X	WO,A,94 09010 (SANDOZ) 28 April 1994 cited in the application see claim 1	1-9
X	US,A,5 262 423 (KAO) 16 November 1993 see claim 1	1-9
X	US,A,5 258 389 (GOULET) 2 November 1993 cited in the application see claim 1	1-9
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1995	09.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gettins, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 221 670 (CAUFIELD) 22 June 1993 see claim 1	1-9
X	US,A,5 151 413 (CAUFIELD) 29 September 1992 cited in the application see claim 1	1-9
X	US,A,5 120 842 (FAILLI) 9 June 1992 cited in the application see claim 1	1-9
X	US,A,5 118 678 (KAO) 2 June 1992 cited in the application see claim 1	1-9
X	US,A,5 118 677 (CAUFIELD) 2 June 1992 cited in the application see claim 1	1-9
X	WO,A,92 05179 (AMERICAN HOME PRODUCTS CORPORATION) 2 April 1992 cited in the application see claim 1	1-9
X	US,A,5 100 883 (SCHIEHSER) 31 March 1992 cited in the application see claim 1	1-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/EP 94/04191

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US-A-5310901	10-05-94	NONE		
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		WO-A-	9410176	11-05-94
US-A-5258389	02-11-93	NONE		
US-A-5221670	22-06-93	US-A-	5358944	25-10-94
		US-A-	5378696	03-01-95
		AU-B-	653175	22-09-94
		AU-A-	8659991	15-04-92
		CA-A-	2051781	20-03-92
		EP-A-	0549727	07-07-93
		HU-A-	65763	28-07-94
		JP-T-	6501012	27-01-94
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		JP-A-	5078377	30-03-93
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		EP-A-	0515140	25-11-92
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		NZ-A-	242778	26-07-94
WO-A-9205179	02-04-92	AU-B-	653175	22-09-94

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/EP 94/04191

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9205179		AU-A-	8659991	15-04-92
		CA-A-	2051781	20-03-92
		EP-A-	0549727	07-07-93
		HU-A-	65763	28-07-94
		JP-T-	6501012	27-01-94
		US-A-	5358944	25-10-94
		US-A-	5378696	03-01-95
		US-A-	5221670	22-06-93
		CA-A-	2051782	29-03-92
		US-A-	5130307	14-07-92
US-A-5100883	31-03-92	NONE		

1996

Therapeutic Immunology

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Therapeutic Approach to Organ Transplantation

Terry B. Strom and
Manikkam Suthanthiran

Organ transplantation is firmly established as the preferred treatment for many patients suffering from end-stage renal, cardiac, pulmonary, or liver disease or insulin-dependent diabetes mellitus. Nonetheless, no absolute consensus has developed on how to achieve optimal immunosuppression, and many individual centers using somewhat different protocols report excellent graft and patient survival.

Immunologic considerations, including antirejection therapy, are organized around a few general principles. The first consideration is careful patient preparation, and, in the circumstance of renal transplantation, selection of the best available ABO-compatible, human leukocyte antigen (HLA) match in the event that several potential living donors are available for organ donation. Second is a multitiered approach to immunosuppressive therapy similar in principle to that used in chemotherapy; several agents are used simultaneously, each of which is directed at a different molecular target within the allograft response (Fig 36.1, Table 36.1). Additive-synergistic effects are achieved through application of each agent at a relatively low dose, thereby limiting the toxicity of each individual agent while increasing the total immunosuppressive effect. Third is the principle that higher immunosuppressive drug doses or more individual immunosuppressive drugs are required to gain early engraftment and to treat established rejection than are needed to maintain immunosuppression in the long term. Hence, intensive induction and lower dose maintenance drug protocols are used. Fourth is careful investigation of each episode of post-transplantation graft dysfunction, with the realization that most of the common causes of graft dysfunction, including rejection, can (and frequently do) coexist. Successful therapy, therefore, often involves several simultaneous therapeutic maneuvers. The fifth principle involves the appropriate reduction or withdrawal of an immunosuppressive drug when that drug's toxicity exceeds its therapeutic benefit.

PRETRANSPLANTATION TRANSFUSIONS

Although pretransplantation random whole blood transfusion was a powerful adjunct to transplant therapy when cyclosporine was not available, the short-term benefits of random transfusion have recently been more difficult to demonstrate in the cyclosporine era. There is no agreement concerning the role of donor-specific transfusions (DST) for recipients of HLA-mismatched living, related donor renal transplants. Occasionally, DST produces adverse presensitization to the donor. Because these sensitized patients cannot undergo transplantation with tissues procured from the transfusion donor, many units do not use routine DST. Owing to the powerful tolerizing effects of DST in experimental models, there are several active clinical trials evaluating various forms of pre- and perioperative donor blood element infusions into graft recipients as a therapeutic modality.

THERAPY DESIGNED TO PREVENT REJECTION

Antirejection protocols are aimed at interrupting several discrete stages in the immune activation pathway, leading to allograft rejection (1–3). When possible, selection is undertaken using HLA matching to minimize histoincompatibility between donor and recipient (4, 5). All post-transplantation immunosuppressive protocols use at least two agents, each directed at a discrete site in the T-cell activation cascade (see Fig 36.1, Table 36.1).

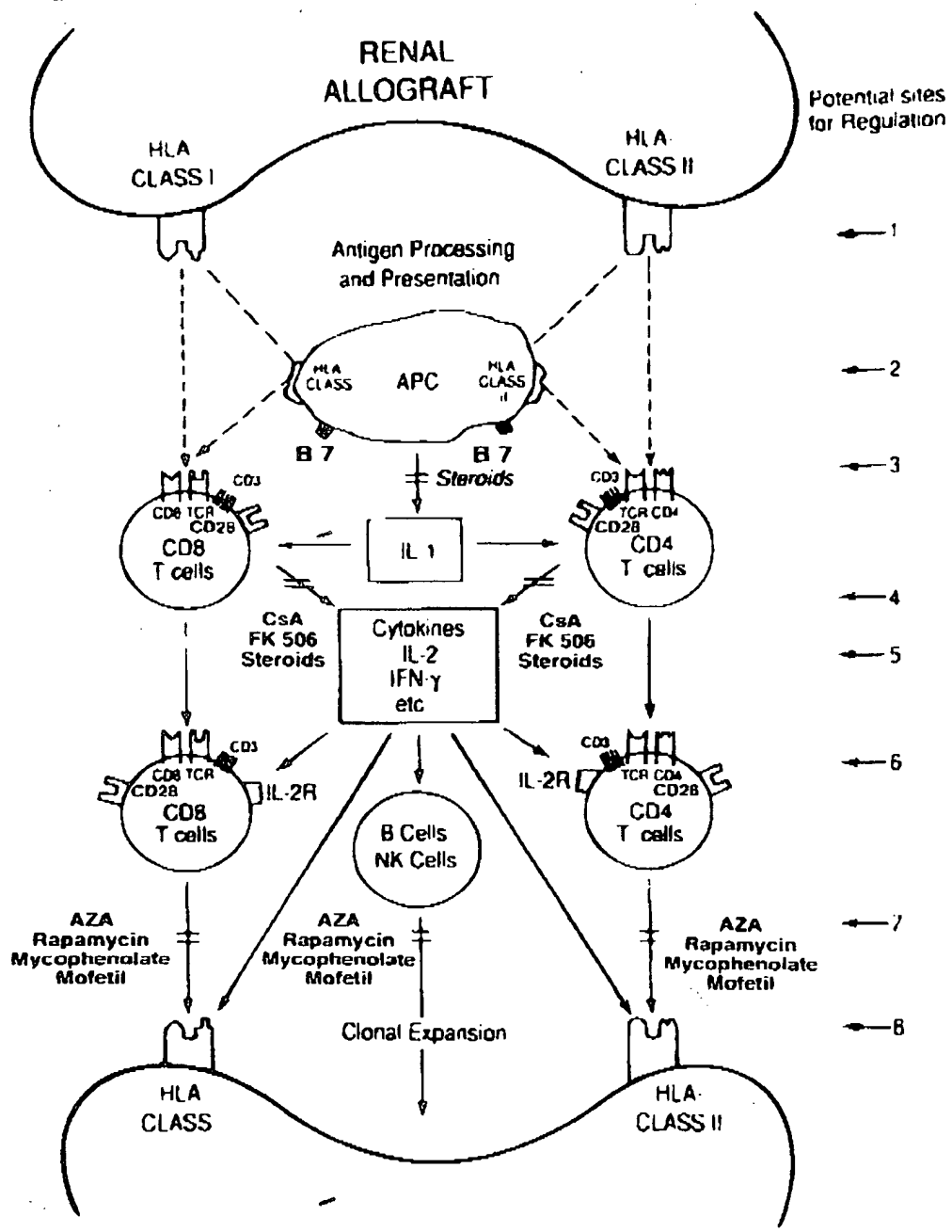

FIGURE 36-1 *The antiallograft response. Schematic representation of human leukocyte antigen (HLA), the primary stimulus for the initiation of the antiallograft response; cell surface proteins participating in antigenic recognition and signal transduction; contribution of the cytokines and multiple cell types to the immune response; and the potential sites for the regulation of the antiallograft response. Site 1: minimizing histoincompatibility between the recipients and the donor (e.g., HLA matching); site 2: prevention of monokine production by antigen-presenting cells (APCs) (e.g., corticosteroids); site 3: blockade of antigen recognition (e.g., OKT3 monoclonal antibodies); site 4: inhibition of T-cell cytokine production (e.g., cyclosporine [CsA]); site 5: inhibition of cytokine activity (e.g., anti-interleukin [IL]-2 antibody); site 6: inhibition of cell cycle progression (e.g., anti-IL-2 receptor antibody); site 7: inhibition of clonal expansion (e.g., azathioprine); site 8: prevention of allograft damage by masking target antigen molecules (e.g., antibodies directed at adhesion molecules). (HLA-class 1 = HLA-A, B, and C antigens; HLA-class 2 = HLA-DR and DQ antigens; AZA = azathioprine; IFN-γ = interferon-γ; NK = natural killer; TCR = T-cell receptor.)*

Table 36.1. Mechanism of Action of Immunosuppressants

AGENT	MODE OF ACTION
Cyclosporine-FK506	Blocks Ca^{2+}-dependent T-cell activation pathway via binding to calcineurin
Corticosteroids	Blocks cytokine gene expression
Azathioprine	Inhibits purine synthesis
Mycophenolate mofetil	Inhibits a lymphocyte-specific guanosine synthesis pathway
Rapamycin	Inhibits the response of antigen-activated lymphocytes to growth factors

IMMUNOPHARMACOLOGY OF ALLOGRAFT REJECTION

Cyclosporine and tacrolimus (FK506)

Cyclosporine, a small neutral hydrophobic cyclic peptide of fungal origin, and tacrolimus (FK506), a water-soluble macrolide lactone produced by *Streptomyces tsukubaenis*, block the Ca^{2+}-dependent T-cell activation pathway (6–9). Oral doses of both agents are erratically absorbed. Insofar as absorption of cyclosporine, but not FK506, requires bile, the absorption of FK506 is not influenced by clamping a T-tube. In this limited respect, treatment of liver transplant recipients with FK506 provides superior bioavailability. The immunosuppressive effects of cyclosporine and FK506 are dependent on the formation of heterodimeric complexes that consist of the native compound (cyclosporine or FK506) and its respective cytoplasmic "immunophilin" receptor protein, cyclophilin (10) or FK-binding protein (FKBP) (11, 12). Both cyclosporine-cyclophilin and FK506-FKBP complexes bind calcineurin, a calcium- and calmodulin-sensitive phosphatase, and inhibit its enzymatic activity (see Fig 36.1; Table 36.1) (13–15). Cyclosporine-FK506–mediated inhibition of calcineurin's phosphatase activity prevents the dephosphorylation of cytoplasmic NF-AT and thereby impedes subsequent import of this deoxyribonucleic acid (DNA)-binding protein into the nucleus (7, 8). Cyclosporine-FK506 inhibits the expression of not only NF-AT (16, 17) but also the activities of other DNA-binding proteins such as NF-kB and activated protein-1 (AP-1) factors (18–20). The phosphorylation status of transcription factors affects not only their nuclear import but also their DNA-binding ability and interaction with the cellular transcriptional machinery (e.g., c-*jun*) (21).

Cyclosporine-FK506 inhibits activation of several cytokine genes, including the interleukin (IL)-2, IL-4, and interferon (IFN)-γ genes; however, this activity does not totally account for the antiproliferative effect of cyclosporine-FK506 on activated T cells. Inhibition of expression of proto-oncogenes (e.g., H-*ras*, c-*myc*) as well as prevention of expression of receptors for cytokines (e.g., the IL-2 alpha chain receptor) might also be quite important in this regard (22, 23).

It is also significant that cyclosporine, in striking contrast to its inhibitory activity on the induced expression of IL-2, enhances the expression of transforming growth factor-β (TGFβ) (24). Because TGFβ is a potent inhibitor of IL-2–stimulated T-cell proliferation and generation of antigen-specific cytotoxic T lymphocytes, enhanced expression of TGFβ is likely to contribute to the immunosuppressive activity of cyclosporine. This novel effect of cyclosporine suggests also a mechanism for some of the complications (e.g., renal fibrosis) of cyclosporine therapy since TFGβ is a fibroblast growth factor (25).

Corticosteroids

Corticosteroids were first used in clinical transplantation to reverse acute rejection reactions in patients treated with maintenance doses of azathioprine. It is now customary to use modest doses of a corticosteroid in maintenance protocols that also use cyclosporine or tacrolimus with or without azathioprine. A short course of high doses of corticosteroids is often used to treat acute rejection episodes. Corticosteroids inhibit T-cell proliferation, T-cell–dependent immunity, and cytokine gene transcription (including IL-1, IL-2, IL-6, IFNγ, and tumor necrosis factor-α genes) (see Fig 36.1, Table 36.1) (26–28). Although no individual cytokine can totally reverse the inhibitory effects of corticosteroids on mitogen-stimulated T-cell proliferation, a combination of cytokines is effective (29).

Some cytokine genes possess a glucocorticosteroid response element in the 5′ regulatory region that serves as a target for the heterodimeric complex formed by the association of corticosteroids with the intracellular glucocorticosteroid receptor protein. Binding of this complex to the glucocorticosteroid response element can, in theory, block gene expression. Blockade of IL-2 gene transcription, however, involves impairment of the cooperative effect of several DNA-binding proteins (30), although the IL-2 gene does not possess a glucocorticosteroid response element.

There are several additional mechanisms by which glucocorticoids might block T-cell activation. Glucocorticoids can block expression of numerous genes through the noncovalent association of the interaction of the activated hormone-receptor complex with the c-*jun*/c-*fos* heterodimer (AP-1) (31); c-*jun* and

-*fos* heterodimers bind to the AP-1 site of the promoter of many cytokine genes. In keeping with this observation, glucocorticoids interfere with IL-2 gene expression through prevention of nuclear transcription factors binding to the AP-1 and NF-AT sites (32).
Glucocorticoids also inhibit the pretranscriptional calcineurin-dependent pathways for T-cell activation (33). Inhibition by corticosteroids of cytokine production represents an important rationale for its usage in the control of the antiallograft response (see Fig 36.1, Table 36.1).

Azathioprine

Azathioprine is the 1-methyl-4-nitro-5-imidazolyl derivative of 6-mercaptopurine (34, 35). This purine analogue functions as a purine antagonist and inhibits cellular proliferation (see Fig 36.1, Table 36.1).
Allopurinol blocks the catabolism of azathioprine, causing a dramatic increase in bone marrow suppression. Azathioprine is often used in conjunction with cyclosporine or tacrolimus and corticosteroids in maintenance protocols. Although application of azathioprine diminishes the incidence and intensity of rejection episodes, it is not valuable in the therapy of ongoing rejection. An agent now being tested in late clinical trials, mycophenolate mofetil, blocks purine metabolism through its inhibitory effect on inosine monophosphate dehydrogenase, an enzyme in the de novo purine biosynthetic pathway (36, 37). The effects of mycophenolate mofetil on purine metabolism are rather selective for activated lymphocytes (38). As a consequence, mycophenolate mofetil may replace azathioprine in some drug regimens (39).

OKT3 monoclonal antibody

The multimeric CD3 complex proteins are noncovalently associated to the alpha and beta chains of the T-cell receptor (TCR) for antigen. This complex is expressed on the surface of all functionally competent T lymphocytes. OKT3 binds to the sigma-chain of the CD3 complex; OKT3 binding to T cells leads to modulation of all components of the TCR-CD3 complex from the T-cell surface, either by shedding or internalization (40). Moreover, T cells virtually disappear from the peripheral blood after the administration of OKT3 monoclonal antibody (mAb).

Maintenance immunosuppressive regimens

The basic immunosuppressive protocol used in most transplant centers involves the use of multiple drugs (usually cyclosporine or FK506 plus corticosteroids with or without azathioprine), each directed at a discrete site in the T-cell activation cascade (see Fig 36.1, Table 36.1) and each with distinct side effects (35). Cyclosporine, FK506, azathioprine, corticosteroids, as well as mycophenolate mofetil are already approved by the Food and Drug Administration, and the clinical efficacy of rapamycin (an agent that inhibits the proliferative signal imparted by IL-2, IL-4, or IL-7 to antigen activated T cells) is being explored in clinical trials.

ALTERNATIVE APPROACHES

Sequential immunotherapy

Following the lead of Soulillou et al (41), a number of other centers have adopted an alternative approach to antirejection prophylaxis. OKT3 mAb or polyclonal antilymphocyte antibodies are used in many transplant centers as induction therapy in the immediate post-transplantation period (42). Administration of antilymphocyte globulin or OKT3 in conjunction with corticosteroids, azathioprine, and greatly reduced doses of cyclosporine-FK506 are applied in the immediate post-transplantation period. This protocol establishes an immunosuppressive umbrella that enables initial engraftment without immediate use of high-dose cyclosporine-FK506 during the early post-transplantation period and enables engraftment without use of high-dose cyclosporine-FK506 in the critical early post-transplantation days, during which time renal grafts are highly sensitive to the nephrotoxic effects of cyclosporine-FK506. The incidence of early rejection episodes is reduced by the prophylactic usage of antilymphocyte antibodies. Any incipient rejection is treated by the use of what we view as essentially antirejection strategies to induce immunosuppression. This protocol is particularly beneficial long term for patients at high risk for immunologic graft failure (e.g., broadly presensitized or retransplant patients) (43).

Therapy designed to treat established rejection

Low-dose prednisone, cyclosporine-FK506, and azathioprine maintenance drug therapy is effective in the prevention of acute cellular rejection; each drug blocks a different facet of T-cell activation. Their proximal sites of activity, however, render low-dose prednisone, cyclosporine-FK506, and azathioprine ineffective in blocking the activity of already activated T cells or late-acting elements of the allograft response that no longer require helper T-cell input. Thus, these agents do not readily abrogate an established acute rejection episode or totally prevent chronic rejection. Treatment of established rejection requires the use of agents that act against already fully activated T cells. In contrast, high-dose corticosteroids, polyclonal antilymphocyte antibodies, or OKT3 are often successfully used as therapies for the treatment of acute cellular rejection.

Approximately, two thirds of acute cellular rejections will respond to high-dose corticosteroid boluses (1). Steroid-sensitive rejection episodes are typically charac-

terized by a dense infiltration of T cells in the medullary regions of the graft. We often treat the first kidney allograft rejection episode with 1 gm of intravenous methylprednisolone daily for 3 consecutive days. The mechanism by which corticosteroids act to reduce the intensity of leukocytic infiltration in a rejecting allograft has not been fully elucidated; however, release of numerous cytokines is blocked by high-dose steroids, and T-cell trafficking patterns are altered.

OKT3-treated T cells lose their antigen receptor proteins and become literally blinded to the presence of the allograft; thus, rejection abates. OKT3 is superior to standard high-dose corticosteroid therapy for reversing kidney allograft rejection (90% versus 70% success) (40). More than 90% of first rejections and a high percentage of second rejections respond to OKT3 therapy. Nonetheless, OKT3 is often reserved as treatment for corticosteroid-resistant rejection episodes. As antirejection treatment, OKT3 is given as a daily 5-mg intravenous bolus for 5 to 10 consecutive days.

Although prophylactic administration of OKT3 to patients in the immediate post-transplantation period is well tolerated, administration of the first and occasionally second dose of OKT3 to patients treated for ongoing rejection often causes a "capillary leak" syndrome that can lead to severe adult respiratory distress syndrome–type pulmonary edema, hypotension, or aseptic meningitis (1, 2, 44). This syndrome is caused by the release of lymphokines from the OKT3-targeted activated T cells. Because of these troublesome symptoms as well as additional expense, we reserve OKT3 therapy for steroid-insensitive rejection episodes. Subsequent doses are well tolerated. Approximately 75% of patients develop immunoglobulin (Ig)G or IgM anti-idiotype or anti-isotype antibodies against OKT3. Azathioprine at doses of 1 to 2 mg/kg/day and prednisone at 30 mg/day are used to limit the frequency and delay the onset of occurrence of host anti-OKT3 antibodies. OKT3 is not efficacious in patients who have developed high-titer anti-idiotypic antibodies against OKT3. Anti-isotypic antibodies do not neutralize the immunosuppressive properties of OKT3.

Polyclonal or antilymphocyte antibody preparations are derived from animals immunized with human lymphocytes. The antibodies are directed against both lymphocyte-specific antigens and more broadly expressed antigens. More than 80% of steroid-resistant first rejection episodes will respond to these polyclonal antibodies. Patients are skin tested with 0.1 mg of a 1:1000 dilution of polyclonal antilymphocyte antibodies before administration of the first dose and pretreated before each dose with diphenhydramine and steroids. Antilymphocyte antibodies, often at a dosage of 10 to 15 mg/kg, are administered daily by slow intravenous infusion over 4 to 8 hours for 10 to 14 days. Adverse reactions include anaphylaxis, hemolysis, thrombocytopenia, neutropenia, dyspnea, chills, fever, hypotension, chemical phlebitis, pruritus, serum sickness, and chest, flank, and back pain. Unlike the complications noted with the first dose of OKT3, the severity of anaphylactoid side effects to these polyclonal antilymphocyte preparations can increase with subsequent doses. Frank anaphylaxis can occur anytime during treatment. The use of polyclonal antilymphocyte antibodies has decreased in the United States because OKT3 is less toxic and comparably effective in reversing rejection.

We rarely treat a kidney transplant recipient for more than three rejection episodes in the early post-transplantation period because third and fourth rejections tend to be vasculitic forms that are therapeutically resistant, and the risks to the patient from zealous immunosuppression are unacceptably high by that point. In contrast, patients with cardiac allografts who will die with the cessation of cardiac function are treated more vigorously because complete rejection, in the absence of retransplantation, is fatal.

Although current drug protocols are far superior to those used a decade ago, the situation is far from ideal. Most allografts eventually succumb to chronic rejection. Long-term therapy is mandatory. We anticipate clinical application, in the near future, of more refined immunosuppressive regimens: new drugs, humanized mAbs, and fusion proteins that target discrete steps in antigen recognition, signal transduction, and effector immunity. We are also optimistic regarding the inducibility of antigen-specific tolerance in the clinical setting, but a delivery date cannot yet be guaranteed.

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